Epic studies of dietary restriction, feeding behaviour and insulin signalling in the fruit fly, *Drosophila melanogaster*.

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Declaration

I confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

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Abstract

Dietary restriction (DR) extends lifespan in many organisms, through unknown mechanisms that may or may not be evolutionarily conserved. Currently, different diets and techniques exist in different laboratories to implement DR in the fruit fly, *Drosophila melanogaster*. Furthermore, DR is often implemented by food dilution, a technique that potentially enables flies to compensate for reduced nutrition by increasing their food intake. Thus, findings from DR experiments may not be strictly comparable because of these methodological differences and uncertainties. In this thesis, I examined the ability of DR to extend life in *D. melanogaster* with different genotypes and on different diet compositions. I demonstrate that many of the different diets used in different laboratories are inappropriate for DR and propose an optimised protocol to insure that DR studies are standardised. I also demonstrate, by combining two available feeding assays, that *D. melanogaster* do not compensate their food intake when exposed to DR by food dilution. The optimised feeding assay in this thesis provides a useful tool for researchers to measure fly total feeding, which is fundamental to studies involving their behaviour, nutrition and/ or drug administration. I demonstrate with this method that food intake is not altered in the lifespan extending mutation *chico*, the receptor substrate of the insulin/insulin-like signaling (IIS) pathway. IIS is a highly conserved pathway responsible for growth, development, stress resistance, metabolic homeostasis, reproduction, and recently, single gene mutations in the pathway have been shown to extend lifespan. One component of the pathway, dAkt, has yet to be established as a regulator of lifespan and fecundity in *D. melanogaster*. Although there is sufficient literature regarding its role in growth and development in fly larvae, it is much less understood in adults. Using viable *dAkt* mutants, I establish that for lifespan benefits to occur, the appropriate level of IIS reduction is required.

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1.1. Introduction to ageing

1.1.1. What is ageing?

Ageing is a complex biological process that is commonly defined as the spontaneous and progressive change in the tissues and organs of the body, which increases the probability that an organism will die for internal reasons and decreases the rate at which it successfully reproduces (Partridge and Barton, 1996; Kirkwood, 2005). Classical evolutionary models of ageing predict that all species eventually succumb to ageing (Hamilton, 1966). However, contradictory studies have recently emerged providing examples of animals and plants that do not show signs of ageing (reviewed in Finch, 2009). These include, the freshwater metazoan *Hydra vulgaris* (Martinez, 1998), the red sea urchin *Strongylocentrotus franciscanus* (Ebert, 2008), the painted turtle *Chrysomys picta* (Congdon *et al.*, 2003), *Sebastes* rockfish (Finch, 1990) and the naked mole-rat *Heterocephalus glaber* (Buffenstein, 2008); all of which show no age-related deterioration either in terms of survival or reproduction rates. Evidence has also been found for negative ageing, where mortality declines and fecundity increases with increasing age after reproductive maturity (Finch, 1990; Vaupel *et al.*, 2004; Baudisch, 2005). Thus, ageing does not occur universally in species.

Despite this fact, maximum lifespan varies greatly among many different species: the adult male mayfly *Baetis bicaudatus* survives for about 24 hours (Edmunds and Waltz, 1996), the gastrotrich *Chaetonotus maximus* 15 days (Balsamo and Todaro, 1988), the fruit fly *Drosophila melanogaster* a few months (Ashburner, 1989), the laboratory mouse 4 years (Finch, 1990), bowhead whales 200 years (George *et al.*, 1999), while a bristlecone pine tree *Pinus longaeva* has been recorded to reach 4800 years old (Currey, 1965).

Differences in lifespan are also apparent between species of similar physiologies. The soil-dwelling nematode *Caenorhabditis elegans* can live up to 3 weeks (Riddle *et al.*, 1997), whilst the parasitic nematode *Loa loa* can survive for up to 17 years (Eveland *et al.*, 1975). Furthermore, isogenic populations of *C. elegans* comprising of genetically uniform individuals, reared in a constant environment and protected from extrinsic mortality were

found to have highly variable lifespans (Finch and Kirkwood, 2000; Kirkwood, 2002). The lifespan of individual nematodes varied by as much as three-fold within the population (**Figure 1.1.1**) and this variability persisted in populations with average lifespans that have been increased by genetic mutations, such as *age-1* (Johnson, 1990) and *daf-2* (Kenyon *et al.*, 1993). Lifespan variability can also arise from the specification or modification of specific genes, for example in honey bees, the lifespan of a queen may last a few years whereas the lifespan of a worker may only last a few months (Finch and Ruvkun, 2001). More recently, the parasitic nematode *Strongyloides ratti*, has been found to survive for up to 403 days in the rat small intestine whereas free-living adults in the soil only survive for up to 5 days (Gardner *et al.*, 2006). Parasitic and free-living female *S. ratti* are morphologically different, yet genetically identical. Thus, the 80-fold difference in their lifespans must largely reflect evolved differences in gene expression. This suggests that inter-specific differences in lifespan may evolve via similar mechanisms.

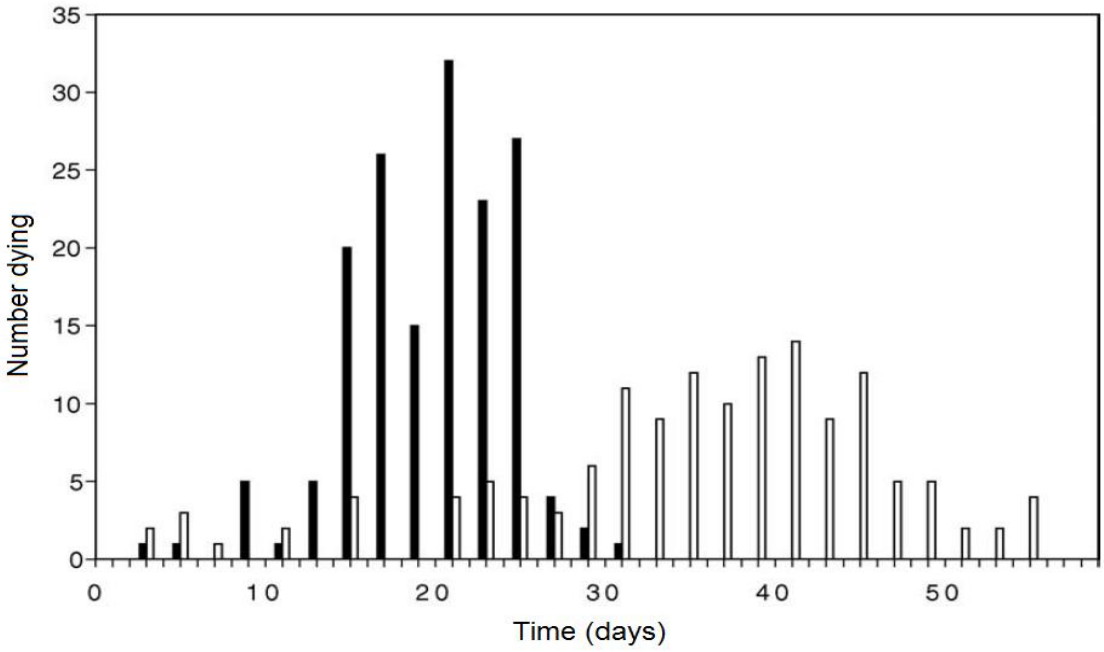

Figure 1.1.1 Lifespan distributions for individual *C. elegans* nematodes in isogenic populations.
A large variation in lifespan exists despite the genetic uniformity of individuals. Wild-type (filled bars) and *age-1* (open bars) strains. Redrawn from (Kirkwood and Finch, 2002).

1.1.2. How is ageing measured?

The most common measurement used in ageing research is drawn from data obtained by a life table for a cohort of individuals of the same age. The life table contains the information concerning the time or age of each of the individuals in a given population.

From these data a survivorship curve can be drawn, showing the percentage of individuals surviving against age for a cohort of same-aged individuals. A derivative of this is the rate of death or mortality rate for the population, which is graphed as the mortality rate – using the natural logarithm. In the wild, the shape of the survival curve usually shows an approximately continuous decrease or constant loss of life over time due to predation, injury, and disease (Finch, 1990) (**Figure 1.1.2A**). When plotted as a rate of death or mortality curve it shows a constant rate of mortality or a straight horizontal line (**Figure 1.1.2B**). This type of curve implies that death is not related to the age of the mature adult but to some cause that does not distinguish between ages such as predation, accidents, or starvation, although such things may in fact be age related. Thus, this population would not be considered to represent a population that is ageing. That is, the probability of dying does not increase as the individuals in the population get older.

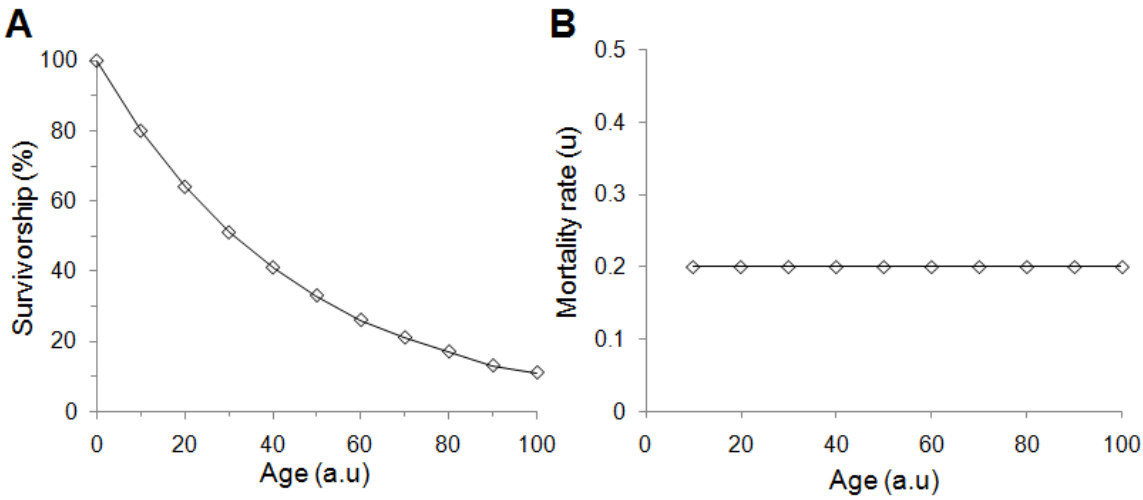

Figure 1.1.2 A population without ageing.
(**A**) An example of a survivorship curve for a population in the wild, based on Finch (1990). (**B**) The mortality rate of the population shown in the survivorship curve.

Another type of survivorship curve that can be observed when a population shows age-dependent death but still does not show 'true' ageing is where the individuals all live to a particular age and then rapidly die-off (**Figure 1.1.3A**). In this instance, the mortality rate here does not show an increase of age-related mortality over a large portion of the lifespan but rather a short period of massive death (**Figure 1.1.3A**). An example population that could create such a survival curve is the Pacific salmon which die immediately after spawning (Finch, 1990).

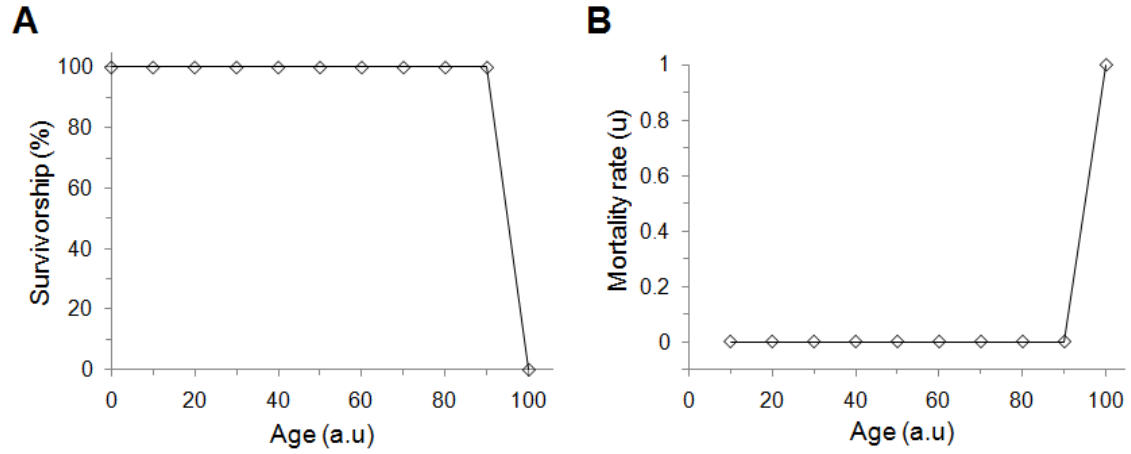

Figure 1.1.3 A population where mortality is age-dependent but is not ageing.
(**A**) An example of a survivorship curve where death is triggered by reaching a particular age. (**B**) The mortality rate of the population shown in the survivorship curve.

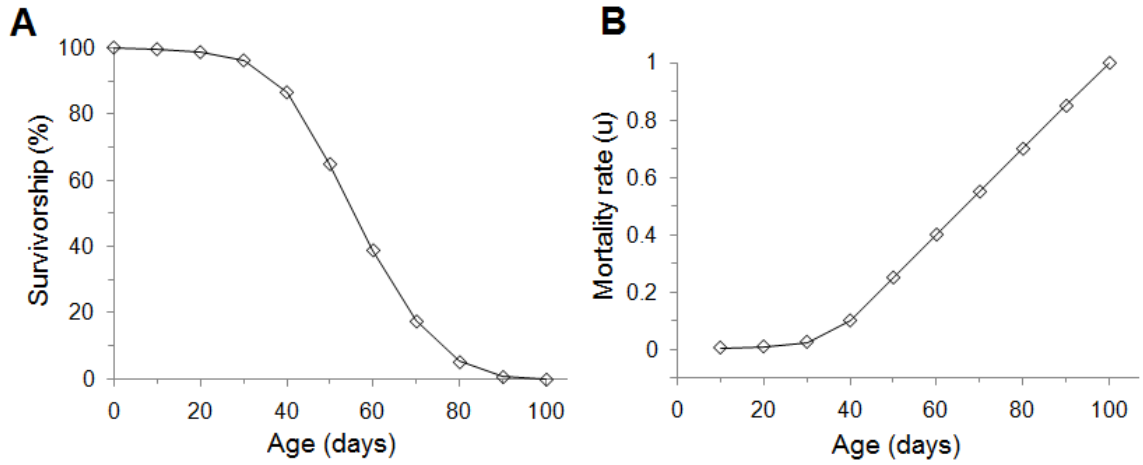

Figure 1.1.4 A population that is showing age-dependent increases in mortality, i.e. 'true' ageing.
(**A**) The survival of *Drosophila melanogaster* at 25°C. (**B**) The mortality rate of the population shown in the survivorship curve.

Figure 1.1.4A-B shows survivorship and mortality curves that represent the true elements of ageing in a population. After an initial early period of low mortality, the initial mortality rate (IMR) which does not including infantile mortality, as individuals get older there is an increasing rate of mortality, or decreasing rate of surviving to the next period of time. This idea of an increase in mortality rate, more specifically, an exponential increase in the rate of mortality with age was first developed in 1825 by the British actuary Benjamin Gompertz (Gompertz, 1825). The rate of increase of mortality is commonly described by reference to the mortality rate doubling time (MRDT), which is the constant time required for the mortality rate to double from any particular initial value. An exponential increase in mortality with age demonstrated not only the increasing

burden of age on survival, but also provided the underpinning for another commonly known phenomenon, species-specific lifespan.

The idea of a species-specific lifespan is that each species has a maximal life expectancy that it does not surpass (**Table 1.1**). For example, laboratory mice live 4 - 5 years and dogs live up to 20 years. The idea was that if mortality rates increase exponentially there will have to be an age beyond which no individuals in the population will be alive. Indeed, the MRDT has been documented in a number of different species (Finch, 1990; Holmes and Austad, 1995; Bronikowski *et al.*, 2002; Foote, 2008), and these differences in the longevities of species are the result of differences in MRDT and not IMR. For example, the rhesus monkey, the dog and the lab rat all have different MRDTs while their IMRs are equivalent, yet their longevities are all different. Interestingly, the IMR can be increased by environmental hazards, such as malnutrition which often results in an increased mortality rate (**Figure 1.1.5.**). This suggests that the environment can only change the susceptibility to death but not the intrinsic rate of ageing.

In recent years, deviations from the exponential increase in mortality and species-specific lifespans have been observed in fruit flies (Curtsinger *et al.*, 1992), Mediterranean fruit flies (Carey *et al.*, 1992) and humans (Manton *et al.*, 1994). Three reasons have been proposed to explain this phenomenon: 1) the exponential increase in mortality is only an approximation to the early part of 'true' mortality; 2) there is an essential change in the ageing process in very late-life; 3) large populations are heterogeneous, and frail individuals in this population are more susceptible to death while any remainders will be predisposed to survive. Therefore in very late-life, the mortality rate is lower because of the lower proportion of frail individuals left in the population (Brooks *et al.*, 1994).

It is thought that a change in the slope of the mortality curve represents a change in the rate of ageing, while a shift in the curve reflects a change in the hazard function without actually changing the rate of ageing (Finch, 1990). Interventions that shift the mortality curve while leaving the slope unchanged may delay or shorten the time of initiation of the ageing process but do not affect the rate at which ageing proceeds once it is initiated. One of the major goals of ageing research has been to look for interventions that alter the slope of the mortality curve; such interventions are thought to represent a change in the rate of ageing and thus provide insights into the normal process of ageing.

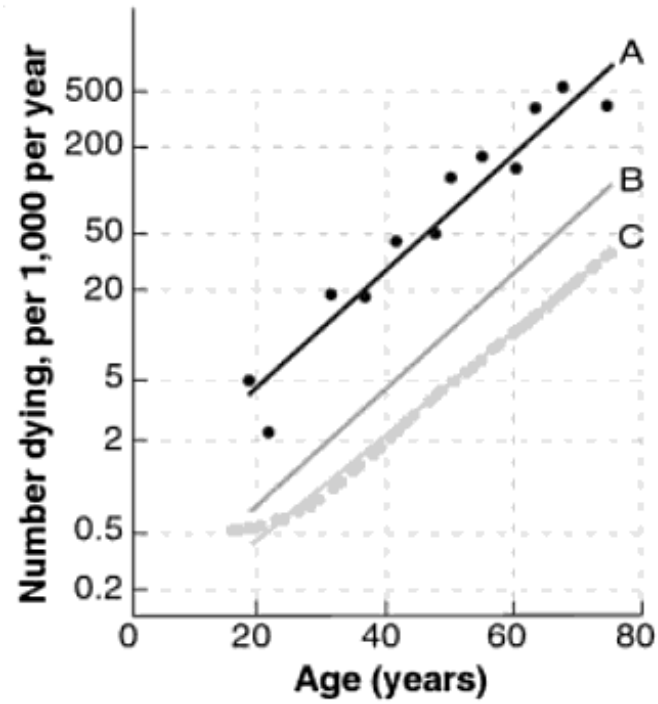

Figure 1.1.5 Mortality rates as a function of age in human populations subjected to prolonged stress. The rate of mortality (i.e. the gradient) remains fairly constant, but the initial mortality rate (i.e. the intercept) changes significantly. Curve A represents Australian prisoners of war held in concentration camps by the Japanese army during 1945; curve B represents civilians in Australia, 1944 – 1945; curve C represents white females in the U.S. 1980 census. (Figure taken from Arking 2006, redrawn from Finch 1990.)

1.2. Model organisms used in ageing research

1.2.1. Introduction

Much of our understanding of human ageing has been built upon the studies of four animal models: the yeast *Saccharomyces cerevisiae*, the nematode worm *Caenorhabditis elegans*, the fruit fly *Drosophila melanogaster*, and the house mouse *Mus musculus*. All share a large collection of genes, proteins, genetic pathways, and metabolic processes with humans. Despite their differences in size, genome and lifespan, these organisms exhibit the same core pathways responsible for growth, reproduction, and coping with stress as in humans (Austad, 2003), as well as exhibiting similar patterns of survival to those of human populations (**Figure 1.2.1**) (Sinclair *et al.*, 1998). These features makes them convenient models for scientific research and comparative studies of interventions that extend lifespan (Liang *et al.*, 2003). In the next section I will briefly describe the uses

of yeast, worm and mouse, before describing in detail the fruit fly *D. melanogaster*, the species used in experiments for this thesis.

Species	IMR/year	MRDT (years)	Maximum life span (years)
Humans			125
Prisoner of war, 1945	0.0070	7.7	—
U.S. female, 1980	0.0002	8.9	—
U.S. female, 1997	0.00004	8.0	—[a]
Baboon females	0.00510	4.8	33[a]
Horse	0.0002	4	46
Rhesus monkey	0.02	15	>35
Domestic dog	0.02	3	20
White-footed mouse	0.06	1.2	8
Lab rat	0.02	0.3	5.5
Lab mouse	0.03	0.27	4.5
Lab gerbil	0.1	0.9	3.8
Pipstrelle bat	0.36	3–8	>11
Herring gull	0.004	6	49
Brush turkey	0.045	3.3	12.5
Bengal finch	0.1	2.5	9.6
Pea fowl	0.06	2.2	9.2
Reeves pheasant	0.02	1.6	9.2
Japanese quail	0.07	1.2	5–8[b]
European robin	0.5	8	12
Starling	0.5	>8	20
Guppy	0.07	0.8	5
Lake sturgeon	0.013	10	>150
Fruit fly	0.01–0.04	0.02–0.04	0.3
House fly	4–12	0.02–0.04	0.3
Honeybee worker			
Winter	<0.001	0.03	0.9
Summer	0.2	0.02	0.2
Soil nematode	2	0.02	0.15
Rotifer	6	0.005	0.10
Nonfeeding moth	10	0.005	0.03

Table 1.1 Representative values of mortality rate coefficients.
Data compiled from Finch (1990), Bronikowski *et al*. (2002) and Holmes and Austad (1995). Table adapted from Arking (2006).

1.2.2. *Saccharomyces cerevisiae*

The yeast, *Saccharomyces cerevisiae* is a useful model organism because it is a unicellular eukaryote, and therefore shares genetic and physical similarities to higher

eukaryotes, including humans. It is an ideal model for understanding the processes at work in human cells because all its genetic information is stored in the nucleus and the mitochondria of the cell, as in humans, and yeast exhibits all the fundamental eukaryote mechanisms – such as mitosis and intracellular transport. It is a unicellular organism that, unlike many complex eukaryotes, can be grown on defined media, enabling the experimenter to have complete control over its chemical and physical environment. Historically, it was one of the earliest genomes to be fully sequenced (Goffeau *et al.*, 1996).

From the perspective of ageing research, it is an inexpensive organism to maintain, has a short lifespan and can be manipulated easily to measure lifespan. Yeast cells have a limited lifespan, an individual cell divides (or buds) a certain number of times after which it assumes a granular appearance and lyses, the equivalent of cell death. There are two methods of measuring ageing in yeast: chronological and replicative; and different labs use either one or the other depending on the particular question asked (Kaeberlein *et al.*, 2007). Chronological life span (CLS) refers to the length of time a non-dividing cell can maintain viability, as defined by its ability to re-enter the cell cycle after a prolonged period of quiescence.

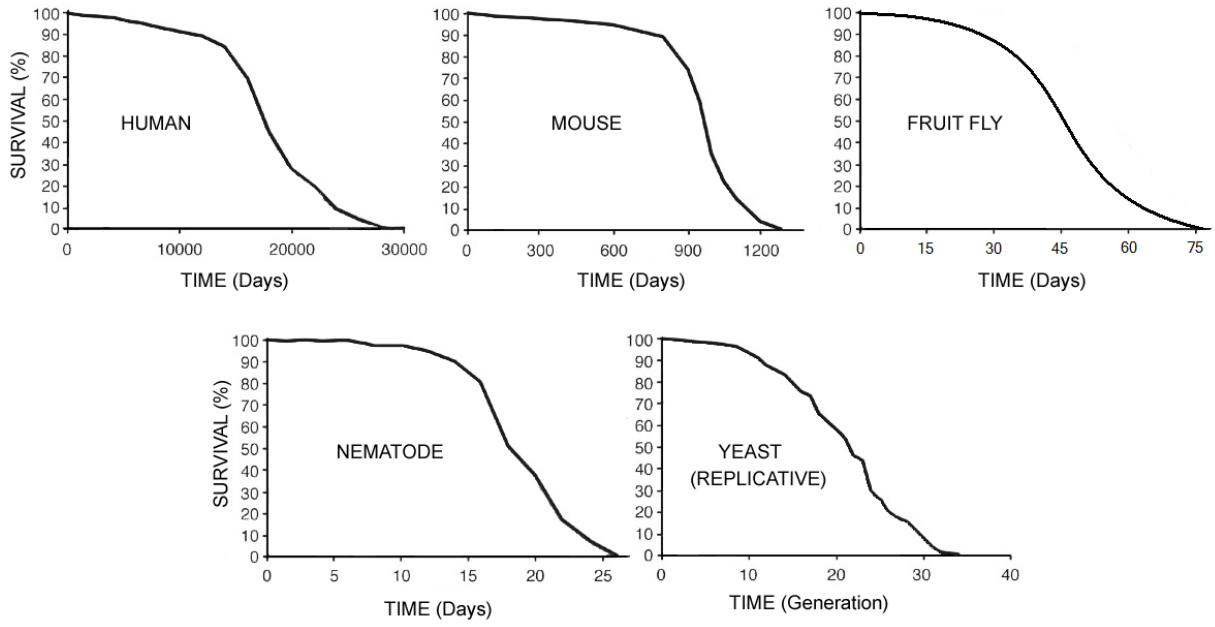

Figure 1.2.1 Population survival curves of different organisms share a similar pattern of decline.

Yeast CLS has therefore been adopted as a model of the viability of post-mitotic cells. Replicative lifespan in yeast is defined by the number of times an individual cell divides (Mortimer and Johnston, 1959), and the mean lifespan of a strain usually falls in the range of 20 to 30 generations or divisions. Cell size increases and the ability to divide decreases with successive rounds of cell division. 'Bud scars' build up on the surface of the mother cell, which can be used as markers of the number of divisions the cell has undergone. As the mother cell divides (or ages), there is a build-up of a 'senescence factor' within the cytoplasm (Kennedy *et al.*, 1994). This is believed to be either extra chromosomal ribosomal DNA circles (ERCs), which is thought to limit the replicative potential of the yeast cell (Sinclair and Guarente, 1997) or oxidatively damaged (carbonylated) and aggregated proteins (Aguilaniu *et al.*, 2003).

Asymmetric cell division occurs in *S. cerevisiae* and the replication potential of the new daughter cells produced is 'reset' to zero, i.e. daughter cells can produce about 30 daughter cells herself. However, this age-asymmetry between mother and daughter weakens in late-life, and daughter cells from senescing mothers also have a reduced replicative lifespan. There may be a critical limit of senescence factors in the mother cell, before transference occurs from mother to daughter during division (Kennedy *et al.*, 1994). Similar to mammalian cells, yeast cells also show a limited replicative potential, and this, together with their large degree of genetic homology with higher organisms, make yeast an important model organism for ageing research.

1.2.3. *Caenorhabditis elegans*

Caenorhabditis elegans, is a nematode worm approximately 1.2mm in size, with a 3-day life cycle and a mean lifespan of approximately 2-3 weeks at 25^{o}C (Riddle *et al.*, 1997). They are generally cultured in agar plates with slow-growing *Escherichia coli* streaked on the surface as a food source. Although the majority of worms are hermaphrodites that are capable of self-fertilisation, about 0.05% of the total individuals in a population are males (Alberts *et al.*, 2008). This offers geneticists the flexibility of combining and removing genes into and from different strains of worms. Furthermore, strains can be stored at -80^{o}C and survive on thawing, thus providing straightforward

long-term storage. There are many mutant strains of *C. elegans* freely available and the full genome sequence has been published (The *C. elegans* Sequencing Consortium, 1998).

C. elegans has the advantage of being a multicellular eukaryotic organism that is simple enough to be studied in great detail. It is transparent, which facilitates the study of developmental processes in an intact organism, and the developmental fate of every single somatic cell (959 in the adult hermaphrodite; 1031 in the adult male) has been mapped out (Sulston and Horvitz, 1977). These patterns of cell lineage are largely invariant between individuals, in contrast to mammals where cell development from the embryo is more largely dependent on cellular cues. The transparent body also enables researchers to monitor gene expression and the localisation of proteins with a tag such as green fluorescent protein (GFP) (Chalfie *et al.*, 1994).

In the field of nematode ageing research, numerous mutations have been identified that alter the rate of ageing, with some individuals living up to five times as long as wild-type worms (Lakowski and Hekimi, 1996). The extensive mutant library amassed over thirty years of *C. elegans* research enables researchers to identify genes important in lifespan determination. Another useful feature of *C. elegans* is that it is relatively straightforward to disrupt the function of specific genes by RNA interference (RNAi) (Fire *et al.*, 1998). Down-regulating the function of a gene in this way can allow a researcher to infer what the function of that gene may be. The nematode can either be soaked in (or injected with) a solution of double stranded RNA (dsRNA), or fed on bacteria expressing the dsRNA, the sequence of which is complementary to the sequence of the gene of interest. Bacterial libraries of strains that express dsRNA of all known *C. elegans* coding sequences are available, and can be used for genome-wide screens of phenotypes of interest (Lee *et al.*, 2003). These features make the nematode worm, an ideal organism to study ageing with.

1.2.4. *Mus musculus* and *Rattus norvegicus*

Rodents are expensive to maintain, difficult to handle and breed, and require more space to house when compared to invertebrate organisms. Their life cycle and lifespan are also significantly longer, with mice being able to breed after 6 weeks and live between 3-5 years (Weindruch and Walford, 1988). Thus, experiments that involve rodents

generally have smaller sample sizes, take longer to complete and require greater preparation time. On the other hand, lifespan experiments with rodents are necessary because they are models for mammalian physiology, and the information obtained can be more easily extrapolated to humans. The genetics of ageing in mice and rats are less well characterised than in invertebrates but since the publication of the genome sequences of both models (Waterston *et al.*, 2002; Gibbs *et al.*, 2004), the information available has increased substantially (Ladiges *et al.*, 2009).

1.2.5. *Drosophila melanogaster*

The fruit fly is a highly regarded model organism in science because of the extensive history and knowledge available from many years of genetic research. This resulted from the initial work of Thomas Hunt Morgan who wanted to identify the physical mechanism at work in inheritance at the turn of the twentieth century. He chose to study the fruit fly because they possess enlarged polytene chromosomes in their salivary glands that can be visualised easily under the light microscope. Furthermore, they are inexpensive and easy to culture, have a high fecundity, short life cycle of 10-12 days, and a lifespan of about 45-60 days at 25^{o}C (Ashburner, 1989).

As early as the 1900s, fruit flies were used to determine the effect ambient temperature had on longevity (Loeb and Northrop, 1917), and these findings formed the basis of Raymond Pearl's 'Rate of Living' hypothesis (Pearl, 1928) (see **1.4.3.**). The hypothesis enjoyed prominence as one of the foremost theories of ageing for nearly 50 years and secured Pearl as one of the founders of biogerontology. In more recent years, studies using *Drosophila* have significantly contributed to the field of ageing research. Parental age effects have been found to influence juvenile fitness traits and the heritability of morphological traits (Parsons, 1962; Beardmore *et al.*, 1975; Hercus and Hoffmann, 2000; Kern *et al.*, 2001). Molecular genetic studies have identified several genes associated with lifespan in *Drosophila* (see **1.6.4**) (Lin *et al.*, 1998; Parkes *et al.*, 1998; Rogina *et al.*, 2000; Clancy *et al.*, 2001; Tatar *et al.*, 2001; Giannakou *et al.*, 2004) and quantitative genetic studies have established *Drosophila* as an ideal model system in which to test evolutionary theories of ageing (see **1.3**) (Rose and Charlesworth, 1980; Luckinbill *et al.*, 1984; Rose, 1984; Partridge and Fowler, 1992; Zwaan, 1995; Nuzhdin *et*

al., 1997). This work is facilitated by the fact that many mutant strains are freely available, many powerful genetic tools exist for the 'fly pusher' and its genome has been fully sequenced (Adams *et al.*, 2000).

1.2.6. *Drosophila* life history and behavioural habits

Drosophila melanogaster is the most commonly used species of the *Drosophila* genus in biological research. The main details of *Drosophila* life history will be described below, while Ashburner (1989) provides a comprehensive summary. Under laboratory conditions, typically a temperature of 25^{o}C and 65% humidity, *D. melanogaster* has a life cycle of about 10 days with three stages of development from egg to adult: embryonic, larval and pupal. Mated females insert their eggs into the surface of the food via an ovipositor with the micropyle and chorionic appendages of the egg exposed in the air. Generally, fertilised eggs are immediately laid, and hatch after about 24 hours of embryonic development. There are three larval instars, and this is the period of development in which all growth occurs. The larva embarks on a 4.5 day bout of feeding which is followed by a 'wandering stage', where it leaves the food medium to search for a suitable area to pupate. In the laboratory, this usually occurs on the walls of vials or bottles the flies are housed in. Metamorphosis takes place during the pupal stage, which typically lasts for another 4.5 days. Adult flies emerge according to a circadian rhythm and females are unreceptive to mating with males for the first 8-12 hours of their lives. This is of great practical importance, because it means that flies separated by their sex during this period will be virgin and can be used in controlled crosses (Ashburner, 1989).

Aside from males being generally smaller than females, the sex of *Drosophila* can be determined in three ways: 1) males possess sex combs, which are black bristles on the forelegs; 2) the male abdomen is darker than the female abdomen and also contains a genital plate that is absent in the female; 3) the male abdomen has only 5 segments compared to 7 segments in the female.

In the wild, fruit flies are attracted to microbes such as yeast growing on the surface of plants and fruit (Carson, 1971; Kimura *et al.*, 1977). Eggs are usually laid on ripened fruit, so that larvae develop as the fruit begins to rot (Demerec, 1950). If a hungry fly lands on an adequate food source, taste receptors on its tarsi will trigger it to extend its proboscis

and begin to feed. During ingestion, liquid food passes through the anterior portion of the foregut and fills the crop, a collapsible food-storage sac (Gelperin, 1971). As the fly continues to feed, stretch receptors in its foregut and abdomen provide negative feedback to the central nervous system to decrease feeding and increase the threshold for subsequent ingestion (Gelperin, 1971). Aside from attraction to food, *D. melanogaster* also have a propensity to fly towards light. Flies cultured in vials migrate towards the side of the tube that is nearest to the brightest source of light (Hadler, 1964).

1.2.7. *Drosophila* nomenclature

In *Drosophila* genetics, the normal fly is called a 'wild-type' and any fly with a genetic mutation is called the 'mutant'. Historically, mutant genes are given names that generally denote the type of resulting phenotype. For example, the *white* mutant has white eyes compared to the wild-type fly, although the *white* gene is responsible for producing the red pigment in eyes. Gene names are always italicised, and capitalised if the phenotype is dominant but not if it is recessive to the wild-type. *D. melanogaster*, *C. elegans* and human protein products are generally fully capitalised, for example, CHICO and FOXO. Abbreviations are sometimes fully capitalised, such as XDH for xanthine dehydrogenase (the product of the *rosy* gene) and are sometimes in mixed case, such as AChE for acetylcholine esterase (the product of the *Ace* gene).

Each mutation is also given a letter code. Thus, in the case of *white*, the code is a lower case *w*. The wild-type allele is denoted by a superscript $^{+}$ over the mutant letter code. For example, w^{+} denotes a wild-type gene for the white-eye trait, meaning it has a normal eye colour (red not white). Different mutant alleles are distinguished by the addition of superscript letters or numbers, such as $chico^{1}$ and $chico^{2}$. Recently, if newly characterised *Drosophila* coding sequences are orthologues of genes already described in a different organism, the convention is to add a 'd' to the original gene name, for example *dFOXO* for the *Drosophila* orthologue of the human *FOXO* transcription factor coding sequence.

1.2.8. Problems with invertebrate model organisms

Experiments with invertebrate model organisms have demonstrated that changes to genes can dramatically result in increases to lifespan (Smith *et al.*, 2007; Piper *et al.*, 2008). In some cases, mean and maximum lifespan are extended by up to five-fold (Lakowski and Hekimi, 1996). Despite evolutionary conservation of many of the pathways regulating lifespan in model organisms, the response of model organisms to interventions might not be predictive for higher organisms such as humans (Kuningas *et al.*, 2008). This may be due to the increased complexity of higher genomes (Long, 2001), for example, a mammalian genome often contains several homologues of a single invertebrate gene with similar or distinct functions and expression patterns. This can obstruct the assessment of the role of a specific candidate gene, because genetic variance in duplicated genes is likely to have less dramatic effects than in the original single gene in invertebrate organisms (Gu *et al.*, 2003; Conant and Wagner, 2004). Therefore, it is crucial to determine whether lifespan-extending alterations are unique to the species studied, or it is evolutionary conserved across taxa (Partridge and Gems, 2002).

There is some discussion about whether lifespan extension in model organisms may be to some extent a laboratory artefact (Partridge and Gems, 2007). Some wild strains of mice are unresponsive to DR (Harper *et al.*, 2006), suggesting that laboratory breeding may select for a robust dietary restriction (DR) response in mice. However, wild-type nematode worms have been found to respond to DR (Sutphin and Kaeberlein, 2008). Insulin signalling mutant worms, *daf-2* and *age-1* are long-lived in relatively benign laboratory conditions, but these mutants actually die sooner than wild-type worms when placed in conditions that resemble their natural environment (Walker *et al.*, 2000; Van Voorhies *et al.*, 2005). The long-lived mutant fruit fly *methuselah* has also been shown to underperform in most cases under conditions that resemble a more natural situation (Baldal *et al.*, 2006).

Before we can rationally evaluate whether interventions can increase human lifespan substantially and healthily, we will need to understand the primary causes of ageing, which leads to the important question of how and why we age. In the next section, I describe studies that have used model organisms to test and clarify our understanding of the evolutionary and mechanistic theories of ageing.

1.3. The evolutionary theories of ageing

1.3.1. Introduction

At first glance, it is surprising that organisms do age. Why is an adult that emerges from the sophisticated process of development unable to simply maintain the condition it has achieved and instead undergoes a gradual deterioration that ends with death (Williams, 1957)? Is there some advantage to ageing that would explain why ageing exists despite the obvious disadvantage for the individual? Evolutionary biologists were the first to pose these questions, and decades of theoretical and experimental work have culminated in two conclusions: ageing does not have a function, and it exists because it is ignored by natural selection (Williams, 1957). In the following sections, I will briefly outline – in chronological order from the life of Charles Darwin – the key ideas proposed to explain how and why ageing may have evolved, and how these ideas have been either rejected or adapted to data gathered from new experiments.

1.3.2. August Weismann and ageing for the needs of the species

The publication of 'The Principle of Population' by Thomas Malthus in 1798 was highly influential to the discoverers of natural selection, Charles Darwin and Alfred Wallace. Malthus proposed that exponentially expanding populations cannot be sustained by the environment and that a high mortality rate would therefore exist in populations as a negative check to growth (Malthus, 1798). To Darwin and Wallace, this inspired them to think that only a small proportion of offspring would survive to reach adulthood, and that there would be a selection pressure that favoured the individuals with the best adaptations (Darwin, 1859). Throughout his career, Darwin described in meticulous detail how natural selection had shaped the anatomy and behaviour of different species, but he never attempted to discuss why ageing occurred in species. Indeed, it may have puzzled him to explain how natural selection could not eliminate ageing, a process which in essence reduces reproductive fitness. Certainly natural selection would select for gene variants that prolonged health and reproductive ability.

It was August Weismann, one of the earliest proponents of natural selection, who first attempted to explain ageing in evolutionary terms. Before the theory of evolution, the

process of ageing was believed to be the equivalent of machinery deteriorating over time through every day usage (see **1.4.2.**). Weismann accepted this, and hypothesised that animals would eventually become defective over time and, perhaps with Malthus's population theory in mind, proposed that natural selection would select for individuals that aged and died, because this would remove them from the population, stopping them competing for resources better saved for the young. Ultimately, the related offspring would gain an advantage (Weismann, 1891). This led him to postulate that reproduction was essential in a world that invariably causes degradation of the individual, and that individuals evolved to grow old for the 'needs of the species'.

The concept of natural selection working at the group level, for the benefit of the species, has since been discounted by today's evolutionary biologists (Maynard Smith, 1964; Williams, 1966; Dawkins, 1976). Indeed, the theory was abandoned by Weismann himself, as he could not see how natural selection could promote phenotypes that reduce fitness (Weismann, 1904). Despite the inaccuracy of his theory of ageing, Weismann was highly influential to the theories that followed because of his concept of ageing as an evolved phenotype. Indeed it is believed that his germ plasm theory (Weismann, 1889), that the body is divided into either germ cells that transmit hereditary information to the offspring, or somatic cells, may have influenced Thomas Kirkwood's disposable soma theory (Kirkwood, 1977). We shall return to Weismann and his theories later in the chapter.

1.3.3. Haldane and the declining force of natural selection

Decades passed after Weismann's theory before the next major attempt was made to explain ageing from the perspective of natural selection. During this period, Mendel's work on genes was uncovered and the theory of natural selection itself was better refined in light of the genetic advances. Subsequently, J.B.S. Haldane, one of the pioneers of genetics, published a key finding relating to natural selection in 1941. In essence, he noticed that the force of natural selection in an individual decreased with increasing age (Haldane, 1941). This conclusion arose from his study of the dominantly inherited disease Huntington's chorea. Sufferers of Huntington's disease are afflicted with many difficulties, which include muscular and neural degeneration and these complications can reduce life

expectancy dramatically. He realised that this disease persisted in the population because symptoms first appeared in the affected around the age of 40, an age when reproduction had already occurred in the majority of individuals. Consequently, the mutation may have been inherited by offspring, and thus, natural selection was powerless to remove it from the population. The fact that this disease was becoming more prevalent was because individuals were surviving to an older age than in the past, when most affected individuals would have died before symptoms would manifest.

Throughout most of Earth's history, the natural conditions experienced by any species (including our own) were exceptionally harsh and unforgiving. Predation, starvation, and other external hazards to survival would have meant that only a small number of individuals would survive to the beginning of the reproductive period and far fewer would have been around long enough to experience the detrimental effects of ageing. Old age would have been the rare privilege of the few, thereby weakening selection's ability to influence the frequency of mutations with effects at late-ages and providing the opportunity for such deleterious alleles to persist in the population. This argument applies both to mutations that reduce survival and to mutations that impair the ability to reproduce successfully at later ages. That late-life is not under strong selection does not necessarily mean that individuals will evolve to be frail when old. For this, it must be proposed that genetic factors with late-life effects accumulate in populations over evolutionary time and that ageing is the cumulative effect of these mutations. But why should mutations that weaken condition late in life accumulate?

1.3.4. Mutation accumulation and antagonistic pleiotropy

It is possible that, of all the mutations that randomly occur in a population, a large number do not have any effect early in life but are exclusively deleterious late in life. As discussed above, such mutations are almost neutral in terms of their fitness consequences. Single mutations generally start off in one or a very small number of individuals; therefore random sampling of alleles in each generation will most often result in these mutations being purged from the population soon after it appears. Sometimes, however, a nearly neutral mutation may end up in a disproportionate number of offspring by chance alone. In the rare instance where this trend continues over many generations,

a neutral allele can increase in frequency and even become fixed (i.e., reach a frequency of 100%) in a population. The idea that the slow accumulation of late-acting mutations over evolutionary time results in organismal ageing is known as the mutation accumulation (MA) hypothesis (Medawar, 1952). It is important to note that MA does not refer to the accumulation of somatic mutations within an individual's lifetime. Rather, it postulates that mutations accumulate in genomes over the course of many generations.

In 1957, George C Williams published a paper that focussed specifically on the trade-off between fitness and late survival. The paper hypothesised that some of the mutations that enhance reproductive success early in life have an associated cost later on. Such mutations would be selected for because of their advantage when animals are young and actively reproducing, which for reasons already discussed would easily compensate for any negative effects late in life (Williams, 1957). This idea was later coined antagonistic pleiotropy (AP) (Rose, 1982). The concept was later extended by the work of Thomas Kirkwood, who proposed a specific mechanism for why genes would have opposite effects at different ages. His 'disposable soma theory' (Kirkwood, 1977) suggested that organisms face trade-offs between reproduction on the one hand and investment in somatic maintenance and repair on the other and that alleles that increase the allocation of resources to the former process thus compromise the latter.

It should be noted that Medawar's MA theory and Williams' AP theory are not mutually exclusive and may even act at the same time. The main difference between the two theories is that MA theory suggests detrimental genes are accumulated over generations passively, while AP theory suggests that genes are actively kept in the gene pool by selection.

1.3.5. Predictions of the evolutionary theories

The evolutionary biology of ageing makes predictions that can be tested experimentally. First, patterns of ageing must be heritable and influenced by genetic factors. The existence of genes that modulate ageing was first shown by laboratory evolution experiments, where artificial selection for late-life performance resulted in long-lived strains of fruit flies (Rose, 1984). More recently, specific genes that modulate ageing have been identified in several species (discussed in **1.6**). Twin studies in humans

estimate that roughly 50% of the variation in human lifespan is attributable to genetic variation (Herskind *et al.*, 1996). These studies and others have definitively established that differences in lifespan and in the rate of ageing between and within species have a significant genetic basis.

A second prediction of the evolutionary biology of ageing is that the level of external risk should influence patterns of age-related mortality and functional decline. This prediction is derived from mathematical models that suggest environmental risks of mortality, such as accidental death or predation, determine the strength of age-specific selection on survival and reproduction (Charlesworth, 1994). For example, conditions under which adult individuals are exposed to high extrinsic mortality will usually lead to a fast decline in the strength of selection with age (Abrams, 1993) and are thus expected to lead to the evolution of earlier ageing. That faster ageing can evolve under conditions of high external risk for adults has been shown in a laboratory evolution experiment with fruit flies (Stearns *et al.*, 2000). Further support comes from the comparison of lifespans of venomous or poisonous animals to animals without chemical protection (Blanco and Sherman, 2005), of opossums living on an island to opossums living on the mainland (Austad, 1993), and of birds to flightless mammals of similar size (Austad and Fischer, 1991). These studies showed that animals that are chemically protected, living on an island, or able to fly, and thus presumably are less likely to fall victim to predation, exhibit longer lifespan in captivity than comparable animals without such protection. One would then predict that flying mammals also age more slowly than equivalently sized non-flying mammals. Indeed, bats live about three times as long as non-flying mammals, even when differences in hibernation patterns are accounted for (Austad and Fischer, 1991).

A third prediction of the evolutionary biology of ageing is that mutations with age-specific effects exist and arise sufficiently often to account for ageing-related decline. A few experimental studies have investigated the frequency of mutations with age-specific effects. Mutation accumulation studies with fruit flies indicated mutations with an age-specific effect on mortality early in life are quite common, but mutations with an age-specific effect on mortality late in life are rare (Pletcher *et al.*, 1998, 1999). In contrast, for fecundity of fruit flies, the clearest age-specific effects are found for late-acting mutations (Leips and Mackay, 2000). Although superficially these data appear to support the necessary qualitative prerequisites of mutational dynamics that would account for ageing

under the classical hypotheses, this issue has yet to withstand rigorous mathematical investigation to determine whether the effects are quantitatively sufficient to account for the differences measured.

1.3.6. Specific tests of mutation accumulation and antagonistic pleiotropy theory

For a number of years, researchers sought ways to quantify the relative importance of the two classic models of ageing described in **1.3.4**. Most of these experiments were carried out with *Drosophila*, some with other insect species. They were largely inconclusive and reinforced the idea that both mechanisms are likely to be important. Both theories assume mutations with a deleterious effect that is specific for late-age; they differ in whether the same mutation is neutral or beneficial early in life. Surely these two theories are two extremes of a range, and their distinction is artificial. At one extreme is MA, where mutations have absolutely no beneficial effect early in life. At the other lies AP, where mutations have substantial effects that manifest clearly, can be measured, and lead to substantial selection for the alleles. Between these two extremes lies a range of mutations with continuously distributed beneficial effects early in life.

It has been argued that if MA was responsible for ageing, then three features would be inherent in an ageing population: 1) *the variation of deleterious and heritable genes (additive genetic variance) should increase with age.* This was because late onset deleterious mutations should accumulate more often in a population the later they act (Hughes and Charlesworth, 1994). Studies with *Drosophila* have produced conflicting reports with some showing that the additive genetic variation decreases (Promislow *et al.*, 1996; Shaw *et al.*, 1999) and some showing it increases with age (Hughes *et al.*, 2002). 2) *The fitness of offspring from closely related parents should decrease with age.* Late onset deleterious mutations are more likely to be shared by related individuals than unrelated ones; therefore the effect of inbreeding depression should be more noticeable in older related individuals than older unrelated individuals. There has been work in *Drosophila* to support this (Hughes *et al.*, 2002), although it has also been pointed out that old individuals are inherently more frail than young individuals and therefore it is no surprise that old individuals are observed to be more affected by inbreeding (Charlesworth and Hughes, 1996). 3) *The death rate should increase sharply shortly after*

the last age of reproduction, when the force of natural selection declines to zero. In practice, although in general death rates increase exponentially with age, there is no sudden increase at very late ages and, if anything, death rates tend to decline (Carey *et al.*, 1992; Curtsinger *et al.*, 1992; Vaupel *et al.*, 1998). This resulted in either adaptations of the MA theory (Charlesworth, 2001) or entirely new theories (Lee, 2003; Vaupel *et al.*, 2004) being proposed.

The AP theory predicts that successful selection for increased longevity should result in decreased vigour in youth. Indeed, selection experiments that increased longevity in *D. melanogaster* resulted in lines that also had reduced fecundity. Conversely, lines with greater mortality exhibit higher fecundity earlier in adulthood (**Figure 1.3.1**) (Luckinbill *et al.*, 1984; Rose, 1984; Partridge and Fowler, 1992; Zwaan, 1995). This rapid mortality has been shown to be a direct consequence of early fecundity, because when early reproduction was abolished by genetic- or X-ray-induced sterilisation, the early mortality phenotype disappeared (Sgro and Partridge, 1999). However, experiments have also shown that long-lived flies lay more eggs over the course of their lifespan compared to short-lived flies which had greater initial early fecundity (Leroi *et al.*, 1994).

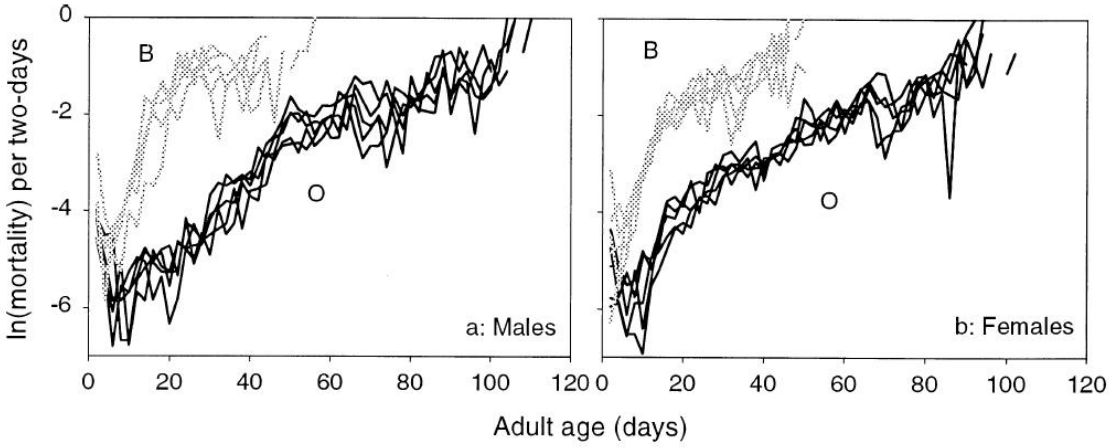

Figure 1.3.1 Two-day mortality rates for two types of *Drosophila* populations.
10 cohorts were sampled from B (early reproducing, grey) and O (late reproducing, black). Individuals in early reproducing populations have increased (steeper) mortality rates than individuals from late reproducing populations. Figure taken from (Rose *et al.*, 2002).

The past two decades have provided experimental evidence for the existence of a balance between lifespan and reproduction. Experiments with *D. melanogaster* suggest trade-offs between longevity and reproduction in both males and females (Zwaan, 1995; Sgro *et al.*, 2000). While a selection regime for flies that favoured late fecundity resulted

in populations with increased lifespan, reduced fecundity and enhanced resistance to a variety of stresses early in life, suggesting that the mechanisms underlying the increase in lifespan involve greater investments in somatic durability. Direct selection for longevity, by exploiting the dependence of the lifespan of fruit flies on temperature, also produced long-lived populations with significantly reduced fertility, underpinning a genetic cause for the trade-off (Zwaan, 1995). Another intriguing intervention, dietary restriction, the reduction of nutrition without starvation, resulted in the extension of lifespan coupled with a reduction in fecundity in a diverse range of animals (reviewed in Weindruch and Walford, 1988). This response may have evolved in an organism as a strategy to deal with famine. During periods of low nutrient availability, a successful strategy would be to shift its resources from reproductive output to somatic cell maintenance in order to increase its chances of survival to more plentiful times, when reproduction would once again increase. A series of point mutations in the *C. elegans* insulin-signaling pathway – regulating metabolism, stress resistance and cell growth – are associated with increase in lifespan of up to 200%, but at the cost of reproductive success (Arantes-Oliveira *et al.*, 2003). Furthermore, removal of germ line precursor cells resulted in a marked increase in worm lifespan (Arantes-Oliveira *et al.*, 2002).

However, a trade-off between reproduction and longevity may not always be obligate. Mutations exist in which longevity was extended with no apparent cost to fecundity (see **1.6**). In nematodes, individuals with mutant *age-1* (Johnson, 1990) and *daf-2* (Kenyon *et al.*, 1993; Gems *et al.*, 1998) alleles showed an extension of lifespan without decreased reproductive ability. Other *daf-2* alleles, which were reported with reduced fecundity, have subsequently been shown to exert their effects on longevity and fecundity at entirely different times during the life of the worm. RNA interference of adult *daf-2* mutants also did not affect fecundity but extended lifespan, although fecundity was reduced when knock-down occurred during development (Dillin *et al.*, 2002). In flies, hypomorphic mutations in the ecdysone receptor (Simon *et al.*, 2003) and over-expression of forkhead transcription factor dFOXO (Hwangbo *et al.*, 2004; Giannakou *et al.*, 2007) all resulted in longevity without reduction of fecundity. While flies that were sterilized by X-ray irradiation or ovo^{D1} mutation showed a normal response to dietary restriction (see **1.5.2.**), an intervention that had been thought to affect ageing partly through its effect on fecundity (Mair *et al.*, 2004). In mice, insulin growth factor-1

receptor (IGF-1R) heterozygotes were also reported to show extended longevity with no reduction in fecundity (Holzenberger *et al.*, 2003). Furthermore, reducing mice fecundity did not necessarily result in increased longevity.

1.4. The mechanisms of ageing

1.4.1. Introduction

Although evolutionary theories of ageing are helpful in explaining why and how ageing evolved, it is uninformative about the specific mechanisms underlying ageing. Indeed, the majority of ageing research in the latter half of the twentieth century has focussed on understanding the underlying mechanism at work in ageing (**Table 1.2**). Although there are many mechanisms proposed, none of the suggestions are mutually exclusive, and ageing may be a result of any or a combination of factors at different life stages. Some of the current or historically important mechanisms are highlighted here:

1.4.2. Wear and tear

The wear and tear theory of ageing is perhaps one of the oldest mechanisms proposed to explain how ageing occurs. It was generally believed that ageing in biological organisms was essentially equivalent to machines breaking-down over time to mechanical damage. Its persistence as a theory was probably down to the everyday observation of infections, wounds and injury encountered by all organisms. Indeed, August Weismann had proposed that the wear and tear in individuals had been the underlying mechanism which resulted in ageing for the 'needs of the species'. Weismann suggested that natural selection was unable to act on somatic cells because they played no part in the transmission of inheritance to the next generation, which is left to germ cells. Thus, somatic cells, which were previously immortal, would gradually 'regress' their immortality, because natural selection was unable to maintain its presence (Weismann, 1891). Ageing was considered as an inherent property of somatic tissues, particularly those that do not show the ability for regeneration or cellular proliferation. Ageing occurs

Mechanism	Major premise and current status
Wear and tear	Ordinary insults and injuries of daily living decrease the organism's efficiency to a sub-vital level. Proven minor role in restricted cases (e.g. loss of teeth leading to starvation), but modern reformulations are part of other theories.
Rate of living	Animals have fixed lifetime energy potentials and lifespan is dependent on the rate that this is used up. Out of favour.
Error catastrophe	Faulty transcriptional and/ or translational processes decrease cell efficiency to a sub-vital level. Out of favour.
Somatic mutation and DNA repair	Somatic mutations alter genetic information and decrease cell efficiency to a sub-vital level. Simplistic interpretations are no longer applicable, but this may be the proximate result of free radical damage. Alternatively, mechanisms which repair DNA damage may vary in efficiency and is positively correlated with lifespan and decreases with age. Known role.
Free radicals and mitochondrial damage	Longevity is inversely proportional to extent of oxidative damage and directly proportional to antioxidant defence activity. Free radicals generated during oxidative phosphorylation may damage the inner membrane and the DNA of mitochondria, decreasing cell efficiency to a sub-vital level. Known role.
Limited replication and telomeres	Cell division is harmful to DNA, and lifespan is determined by the number of divisions a cell can undergo. Ageing is the result of DNA damage caused during cell division. Unknown role and may not be causal.
Waste accumulation	Waste products of metabolism accumulate in the cell and reduce cell efficiency to a sub-vital level. Some compounds are removable from the cell but come at an energy cost, and some compounds can only be decreased by dilution during cell division. Unknown role and may not be causal.
Protein changes	Time-dependent chemical cross-linking and/ or post-translational modification of important macromolecules impairs tissue function and decreases organism efficiency to a sub-vital level. Aggregation of important proteins plays a role in various age-related neurodegenerative diseases. Known role.

Table 1.2 Overview of the mechanistic theories of ageing.
Table adapted from Arking (2006).

simply because a worn-out tissue cannot renew itself forever. The survival potential of individuals should decrease, therefore, with increasing age.

The idea that organisms suffer wear and tear over time is certainly true, and indeed, repair mechanisms do become less efficient with age. However, the idea that inevitable wear and tear is the underlying reason why ageing exists in populations is unfounded. First, animals maintained in an environment that protects them from minor insults and pathologies not only still age but show no improvement in maximum lifespan. Second, there are examples of cell lines which do not show symptoms of wear and tear. These include tumour cells which can infinitely divide (Hayflick, 1977) and cells responsible for the advanced repair systems involved in rapid limb regeneration (Rose, 1991).

1.4.3. Rate of living

The rate of living theory states that longevity is inversely proportional to metabolic rate (Pearl, 1928). The key concept of the theory was that all animals have a limited lifetime of energy potential (LEP), or physiological capacity, and like a star, the faster the energy was used up, the sooner the individual died. This concept, also known as the energy consumption hypothesis, came about from studies that the resting metabolic rate in mammals was inversely proportional to longevity (Rubner, 1908), and that *Drosophila* longevity was inversely proportional to the ambient temperature and therefore metabolic rate (Loeb and Northrop, 1917). The theory relied on the assumptions that longevity was inversely related to both the metabolic rate and the LEP. However, much empirical analyses have contradicted this theory.

LEP and metabolic rate does not always correlate with expected lifespan. For example, humans have twice the maximum lifespan of orangutans and deer but the same level of LEP and metabolic rate (Cutler, 1982), while birds typically have higher metabolic rates than mammals of the same size yet on average live about three times longer (Holmes *et al.*, 2001). Furthermore, the lifetime energetic expenditure per gram body size varied by almost 30-fold among different mammal species (Austad and Fischer, 1991). Studies with long-lived and normal strains of *D. melanogaster* over different ranges of temperatures also found no differences in rates of metabolism even though the long-lived phenotype was still apparent (Arking *et al.*, 1988). Finally, the robust life-extending and health-

preserving effect of dietary restriction (DR) in laboratory rodents previously thought to be partly a result of a reduction in the 'rate of living' was not shown to be associated with reduced metabolic rate. DR resulted in either no change or a slight increase in mass-specific metabolic rate (McCarter and Palmer, 1992; Selman *et al.*, 2005). Thus, the rate of living theory failed to predict differences in ageing rate between species or within species.

1.4.4. Error catastrophe

One of the tenets of molecular biology is that genomic DNA codes for messenger RNA (mRNA) which codes for protein. For this to occur faithfully, a set of specific enzymes and proteins is available to both mediate the synthesis of mRNA by transcription, and the translation of this to protein (these two processes will be abbreviated to 'translation' hereafter). It had been postulated that the machinery for protein synthesis is potentially unstable and errors in the synthesis of proteins may feed back into the translation mechanism, thereby generating further errors (Orgel, 1963, 1970). These errors would accumulate over a lifetime and eventually would lead to a lethal 'error catastrophe'.

To test whether protein error was a cause of ageing, five amino acid analogues (canavanine, ethionine, p-fluorophenylaline, fl-2-thienylalanine and 4-methyl tryptophan) were fed to larval *Drosophila* (Harrison and Holliday, 1967), while in another study, p-fluorophenylalanine was fed to adult male *Drosophila* in sub-lethal doses (Dingley and Smith, 1969). These interventions were insufficient to inhibit normal protein function, prevent metamorphosis, or reduce lifespan. On the other hand, it has been observed that artificially increasing protein errors over many generations in the fungus, *Neurospora crassa*, led to the death of the whole population (Lewis and Holliday, 1970).

This result does question the accuracy of various proof-mechanisms guarding the translation process, and suggests that there may be an optimum level of accuracy. Proofing mechanisms require energy, and there must be some balance between the need for accuracy in the synthesis of macromolecules and the metabolic resources invested to obtain this accuracy. There is no evolutionary advantage in increasing fidelity beyond a certain level, but how far is this level from the critical threshold, below which errors continually increase by a feedback mechanism? Despite the theory falling out of favour to

explain the cause of ageing (Holliday, 1996), there is still much discussion about the stability of the translation machinery and its potential instability (Kirkwood *et al.*, 1984).

1.4.5. Somatic mutations, and DNA damage and repair

This theory hypothesises that ageing is a result of accumulated alterations to somatic DNA, which in turn reduces the function of proteins and therefore cell vitality. There are two ways of altering somatic DNA, either through spontaneous mutation or through damage. Mutation may occur by the substitution, deletion, addition or rearrangement of nucleotides in the strand of DNA, and thus altering (though not always) the coding sequence of genes. DNA damage involves the physical alteration of the double helical structure of the DNA molecule, and this can occur by exposure to radiation, chemicals or free radicals (further described in **1.4.6**). Indeed, it has been observed that X-irradiation can shorten lifespan in animals like mice and *Drosophila* (Lindop and Rotblat, 1961; Lamb, 1963); although contradictory findings have also been observed (Sacher, 1963; Congdon, 1987).

DNA is known to be able to withstand a considerable amount of interference and still be fully functional. This may be partly due to the high level of proofing and repair systems in place to safeguard any mutation or damage to the DNA, and partly because genes generally come in pairs, and consequently, inactivation of one gene would leave the other one intact and fully functional (Szilard, 1959). Therefore, any somatic DNA error that resulted in ageing is more likely to occur in genes that regulated activity and expression of many other genes. Experiments using *Habrobracon*, wasps that have differing number of chromosomes, did not support Szilard's theory (Clark *et al.*, 1963). Here, the lifespan of haploid (single chromosomes in cells) and diploid (pairs of chromosomes in cells) male wasps were measured, and if ageing was a result of error in somatic DNA, then haploid animals should age faster than diploid ones because they lack backup copies of any affected genes. Haploid and diploid males were found to have similar lifespans. However, when both cohorts were subjected to X-ray exposure, haploid males were found to be shorter lived than diploid males, which suggests that the mechanisms involved in the ageing process are different from those involved in decreased lifespan from X-irradiation.

It has been noted that short-lived mice have more abnormal chromosomal damage than long-lived mice (i.e. 20% affected chromosomes at 2 months of age to 80% at 20 months in shorter lived mice, and 10% - 35% in long-lived mice) in liver cells that were recovering from physical damage (Crowley and Curtis, 1963). However, under normal circumstances the steady state level of spontaneous DNA damage or mutation is very low. For example, there are less than 4.3 thymine glycols (a form of DNA damage) per 10^9 bases of DNA in cultured cells (Le *et al.*, 1998), which raises questions regarding studies which involve artificial damage (e.g. X-ray exposure) and subsequent findings of lowered lifespan, because it is possible that artificial damage speeds up disease processes such as malignant tumours, rather than accelerating ageing. Indeed, this is applicable to research relating to DNA repair mechanisms, where mutagen-sensitive flies with defects in DNA repair are significantly shorter lived (Whitehead and Grigliatti, 1993). Additionally, data suggesting that increased copy number of DNA repair gene, *mei-41*, in *Drosophila* led to mild extension of lifespan (Symphorien and Woodruff, 2003), may be a result of protection against disease rather than prevention of ageing.

1.4.6. Free radicals, oxidative and mitochondrial damage

In 1956, Denham Harman developed the free radical theory of ageing (Harman, 1956) after suggestions that free radicals could be toxic agents (Gerschman *et al.*, 1954). Free radicals and oxidants - such as singlet oxygen, O_2^- - also known as reactive oxygen species (ROS), are highly reactive molecules that cause damage to cellular components. Since oxidative damage of many types accumulate with age, the free radical theory of ageing argues that ageing results from the damage generated by ROS (Beckman and Ames, 1998). ROS is generally considered to be a by-product of oxidative phosphorylation and many different types of antioxidants exist to protect against oxidative damage, from vitamins C and E to enzymes such as superoxide dismutase (SOD), catalase, and glutathione peroxidase. SOD catalyses the dismutation reaction:

$$2\ O_2^- + 2\ H^+ \rightarrow H_2O_2 + O_2$$

where H_2O_2 (hydrogen peroxide) is broken down into H_2O and O_2 by catalase or glutathione peroxidise (Halliwell, 2001). Additionally, some enzymes such as methionine sulfoxide reductase A (MSRA) catalyze the repair caused by ROS. The simple existence of enzymes to prevent and repair damage by ROS is a strong indicator that ROS are biologically harmful molecules (Arking, 2006).

Most experimental evidence in favour of the free radical theory of ageing comes from invertebrates. Transgenic *D. melanogaster* over-expressing the cytoplasmic form of SOD (a.k.a. Cu/ZnSOD or SOD1) and catalase had a 34% increase in lifespan (Orr and Sohal, 1994). Furthermore, over-expression of SOD1 in motor neurons (Parkes *et al.*, 1998), and over-expression of MSRA in the nervous system (Ruan *et al.*, 2002) also increased longevity. In *C. elegans*, deletion of SOD1 mildly reduced lifespan; over-expression of the mitochondrial form of SOD (a.k.a. MnSOD or SOD2) and SOD1 extended lifespan minimally; and deletion of MnSOD alone did not affect lifespan (Doonan *et al.*, 2008). This suggests that the role of ROS in nematode ageing is modest, and reflects findings that suggest that the influence of SOD1 and catalase in *Drosophila* ageing might have been overestimated because it only took into account short-lived strains (Orr *et al.*, 2003).

Experiments that fed mice antioxidants of either a single compound or a combination of compounds were able to decrease oxidative damage and increase average longevity of animals but none of them delayed ageing (Comfort *et al.*, 1971; Holloszy, 1998; Saito *et al.*, 1998). Correlations between rate of ageing and antioxidant levels in mammals are, if they exist, very weak (Sohal and Weindruch, 1996). Furthermore, over-expression of SOD1 has been found not to increase mouse longevity (Huang *et al.*, 2000), suggesting the role of ROS in ageing is not well conserved across different species.

Another proposed role of ROS in ageing comes from the fact that the majority of it originates in mitochondria (the source of the cell's energy) during oxidative phosphorylation. This process has been proposed as inherently 'leaky' (Cadenas and Davies, 2000), where 1-2% of oxygen molecules are converted into superoxide anions (Kimata *et al.*, 1999). Mitochondrial DNA, unlike nuclear DNA, is not protected by histones and thus is more likely to be damaged by these ROS molecules originating in the mitochondrion. This occurrence forms the basis of the mitochondrial theory of ageing. However, due to the practical limitations of measuring ROS production in isolated mitochondria, few studies have accurately measured the levels of hydroxyl radical, the

most reactive and destructive of the ROS molecules. Instead hydrogen peroxide and superoxide anion are measured, which may not reflect what damage is occurring in the cell. Thus, further technical studies of ROS are required to establish its role in ageing.

1.4.7. Limited replication and telomeres

Until the work of Leonard Hayflick in the 1950s and 1960s, it was generally and wrongly believed that the majority of cells in the soma were capable of unlimited replication. Alexis Carrel had shown that heart cells taken from chick embryos were 'immortal' and had kept some growing in culture for over 34 years (Carrel, 1912). The reputation of this work delayed Hayflick's work from gaining prominence. However, it was soon realised that cells actually had a finite limit of replications, and this number is entirely predictable and dependent upon the type of cell it is (Hayflick and Moorhead, 1961; Hayflick, 1965). Nobody knows for sure what caused Carrel's mistaken work; it is probable that the culture medium in Carrel's experiments used chick embryo plasma that was contaminated with a few young isolated cells.

Hayflick's work showed that the majority of cells *in vitro*, with the exception of tumour cells and stem cells, go through three main phases during their lifespan: an initial growth phase where they adapt to the new culture in the original flask, followed by exponential cell proliferation, and finally a phase of non-growth. Using human fibroblast cells, Hayflick discovered that these cells survived an average of 50 divisions and that as cells approach the final terminal phase, the length of time between divisions increased and the cells themselves show a number of degenerative changes. Hayflick proposed that this defined limit of replication (the Hayflick limit) is the mechanism responsible for ageing (Hayflick, 1977).

Following this proposal, Hayflick discovered that suspending cells over a period of time, either in liquid nitrogen or in media which stopped cell division, had no effect on the Hayflick limit, and therefore the critical determinant to the lifespan of cells is the number of cell divisions that has occurred rather than chronological age (Hayflick, 1977). The Hayflick limit has since been shown to be correlated with the lifespan of the animal it originated from (**Figure 1.4.1.**) (Rohme, 1981). Additionally, Hayflick also showed that nuclei taken from old cells and transplanted into young host cells had the same replicative

capability as before and therefore suggests that the lifespan-determining component of the cell was located in the nucleus (Hayflick, 1977).

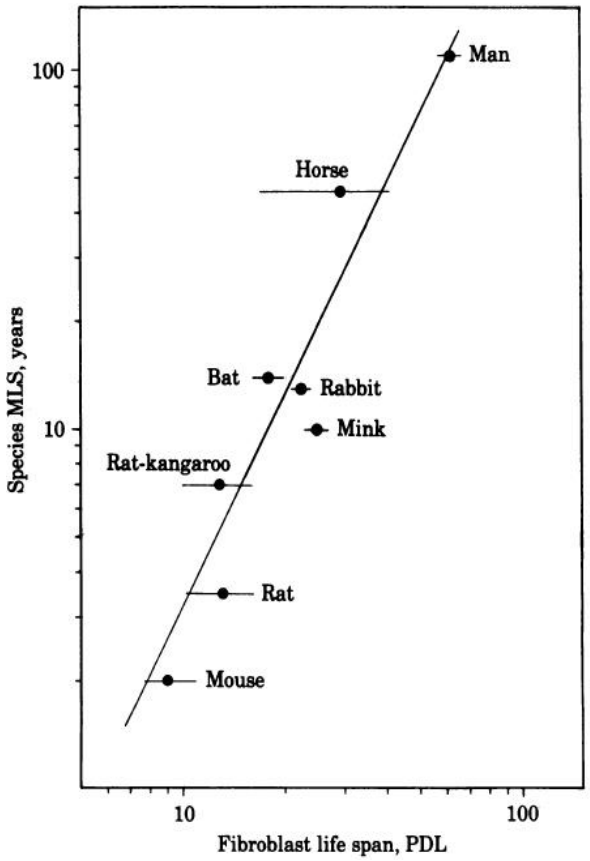

Figure 1.4.1 Graph showing a linear positive relationship between the limit of fibroblast divisions and the mean lifespan for 8 different mammalian species.
Figure taken from (Rohme, 1981).

A new mechanism of ageing has since emerged from these findings. This describes ageing as a result of the depletion of telomeres during cell division, and more specifically during DNA replication (Harley *et al.*, 1990). DNA replication involves DNA polymerase synthesising a new strand of DNA by binding to short RNA primers in a 5' to 3' direction. However, removal of the RNA primers during conventional DNA replication results in the replication of a new strand with a reduced length at the 5' end. Thus, each cell division resulted in an attrition of telomere length (Blackburn, 1991). Telomeres are stretches of repetitive heterochromatin which protect essential coding sequences from this phenomenon. If cells divided without telomeres, they would eventually lose the end of their chromosomes, and the necessary information it contains. As a result, telomeres are compared to aglets, the bits of plastic at the end of shoelaces to protect them from fraying.

Lengthened or stabilised telomeres, a result of increased telomerase (the enzyme which repairs telomeres) are associated with almost every type of immortal or cancer cell line examined (Bryan *et al.*, 1995). Studies of human cerebral grey matter from subjects over 70 years of age have found that longevity is correlated with longer telomeric DNA length (Nakamura *et al.*, 2007); conversely, telomere erosion or down-regulation of telomerase has been linked to higher incidences of mortality (Cawthon *et al.*, 2003) and coronary artery disease (Akbar and Vukmanovic-Stejic, 2007). However, this issue is not clear cut, because telomere shortening is not exhibited in flies, yeast and rodents (Forsyth *et al.*, 2005), which suggests that telomere shortening does not cause ageing universally in the animal kingdom. Indeed, it is known that, apart from cells in the gut and gonads, very little cell division occurs in adult flies (Bozcuk, 1972; Ito and Hotta, 1992) and thus do not suffer the negative effects of cell division described here. More research is therefore needed to justify telomere damage as a cause of ageing.

1.4.8. Waste accumulation

The waste accumulation theory of ageing proposes that cellular ageing is caused by the accumulation of intracellular waste products. One waste product often cited is lipofuscin, pigmented granules that have been observed to accumulate more rapidly in short-lived animals, although no evidence suggest that they are a cause of ageing (Sheehy *et al.*, 1995). However, the idea is that ageing is caused by the accumulation of these waste products which can be highly detrimental to the cell.

Microarray studies in long-lived nematodes have suggested their longevity may be down to the up-regulation of drug detoxification genes (McElwee *et al.*, 2004). These genes remove endobiotic toxic compounds generated during cell metabolism from the cell and therefore prevent macromolecular damage. However, these processes are energetically expensive and a trade-off has been suggested between diverting the resources to removing the accumulated waste products or to reproduction (Gems and McElwee, 2005). Ageing caused by the accumulation of waste products in the cell is a theory that requires more experiments to assess its role, if any, in ageing.

1.5. Non-genetic interventions that extend lifespan in *Drosophila*

1.5.1. Introduction

In this section, some important non-genetic factors that have a direct effect on ageing and lifespan in *Drosophila* is described. These factors include (a) its dietary status (b) its reproductive status (c) the ambient temperature it is maintained in and (d) its exposure to mild stressors, such as heat shock or X-irradiation. Many of these interventions may affect the same or similar physiological functions. For example, the mechanism by which mild irradiation increases lifespan, especially in females, is thought to be due in large part to its effect on reducing fecundity. The dose of radiation that extends lifespan also causes a temporary sterility (Lamb, 1964). Furthermore, females that are sterile, due to a genetic lesion affecting the reproductive system, do not show a further increase in lifespan when irradiated. Some of the effects of heat shock may also be associated with a decrease in female reproduction.

1.5.2. Dietary restriction

Dietary restriction (DR), also known as caloric restriction, is a nutrition restricting method that robustly extends lifespan in a variety of laboratory animals (Weindruch and Walford, 1988). The method was first demonstrated in rats, where animals provided with a restricted but nutritional complete diet had a longer lifespan than those fed a normal unlimited calories diet (McCay *et al.*, 1935). In *Drosophila*, unlike in mammals, DR is applied by reducing the quality rather than the quantity of the nutrients given, either by altering the availability of the live yeast on the food surface or by dilution of all the nutrients in food medium (Chippindale *et al.*, 1993; Chapman and Partridge, 1996).

DR by dilution of live yeast involves flies having free access to varying quantities of live yeast added to a base medium usually containing (but not always) corn flour, sucrose, and agar (Alpatov, 1930). This procedure has yielded conflicting reports with either a 25-30% lifespan extension observed in females (Chippindale *et al.*, 1993) or no lifespan extension found in females (Le Bourg and Minois, 1996). Such differences may stem from variations in experimental procedures such as the use of a different wild-type strain of *Drosophila*

and/ or the use of a different food base medium. However, this method is impractical as it is labour intensive and not fully characterised for fly experiments.

DR by dilution of a yeast-based medium involves diluting concentrations of dead yeast and sucrose dissolved in an agar medium to which flies have free access (Chapman and Partridge, 1996). Despite reducing the quality of the food, the flies still have continuous access since they are living in or on their food, and could therefore eat more to make up for the dilution (Cooper *et al.*, 2004). Measurements of time spent feeding did not differ between flies on DR and fully fed diets (Mair *et al.*, 2005), although a direct study using radioactively-labelled food suggests compensation does occur (Carvalho *et al.*, 2005). However, the majority of *Drosophila* experiments use this method of DR application.

Strong dilution of yeast and sugar in the base medium results in low levels of nutritional availability and can cause malnutrition in flies, which lower their fecundity and lifespan (**Figure 1.5.1**) (Chapman and Partridge, 1996). As the food concentration in the medium increases, the median lifespan and fecundity also increase. However, there is an optimum food level (0.5 SY) for lifespan, where further increases in nutrient availability causes lifespan to decline, even though fecundity continues to increase (Chapman and Partridge, 1996). Fecundity therefore becomes an index of the health of the organism. If increased nutrient intake resulted in the progressive poisoning of an organism, one would predict that both lifespan and fecundity would decline as food intake increased. At high food concentrations although females are shorter lived, they produce more progeny, which suggests that DR does not merely rescue flies from the toxic and/ or pathological effects of over-eating. This data supports the evolutionary trade-off hypothesis, where a balance exists between the ability to maintain high reproductive ability and success, and the maintenance of the individual itself, which in-turn determines lifespan (Kirkwood, 1977).

The lifespan extension response to DR is much greater in females than in males (Magwere *et al.*, 2004) and as yet, it is unknown why this difference occurs. One possible reason is because of the trade-off effect between lifespan and fecundity, with long-lived females generating less eggs than short-lived females (Chapman and Partridge, 1996). This reduction in the cost of reproduction (Sgro and Partridge, 1999) would be greater enhanced in females than in males, because the main life-shortening aspect of reproduction in males is activity and courtship (Cordts and Partridge, 1996).

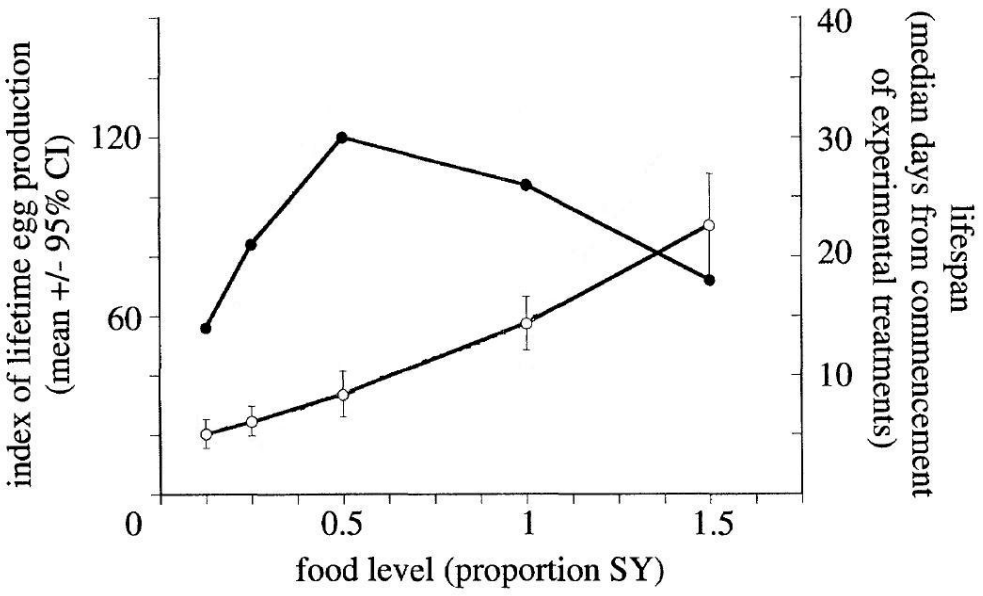

Figure 1.5.1 Index of lifetime reproductive success and lifespan plotted against food level for females continuously exposed to mating males.
Open circles represent egg production and closed circles represent lifespan. Figure adapted from (Chapman and Partridge, 1996).

Increased survival under DR may be a consequence of the removal of the risk of death caused by high reproductive activity (Partridge and Fowler, 1992; Sgro and Partridge, 1999; Barnes *et al.*, 2008). However, transferring flies from a fully fed diet to a DR diet, or *vice versa*, rapidly changes the rate of age-specific mortality to that of the new dietary regime, but does not change the egg-production rates (Mair *et al.*, 2003). This means that the age-specific mortality but not fecundity of DR flies depends on the age and current nutritional status, with nutrition history having no detectable effect. Additionally, the lifespan extending effect of DR in *Drosophila* is dependent upon the reduction of yeast rather than restriction of calories *per se* because reducing the equivalent calorie content by reducing sugar does not extend lifespan (Mair *et al.*, 2005).

DR has also been found to increase longevity in yeast (Jiang *et al.*, 2000; Lin *et al.*, 2002) and nematodes (Houthoofd *et al.*, 2007), however the mechanisms responsible remain to be fully elucidated. Measurements of both oxygen-consumption and heat-production in DR and control flies failed to show DR extends lifespan by reducing the mass-specific metabolic rate (Hulbert *et al.*, 2004). If DR extends lifespan by reducing the levels of oxidative stress, then both the levels of ROS-production and oxidative damage to macromolecules should be reduced by DR. Currently there is little empirical data that tests this theory in fruit flies and results are inconclusive. Measurements of ROS-

production, measured fluorometrically as hydrogen peroxide in mitochondria isolated from *Drosophila* at different ages (Miwa *et al.*, 2003), did not show any significant effect of DR (Miwa *et al.*, 2004). While measurements of mass-specific metabolic rates in DR rodents showed lifespan-extension is not a result of reduced metabolism (McCarter and Palmer, 1992; Selman *et al.*, 2005). Surprisingly, alterations in nutrient consumption may not be the only mechanism that links environmental signals to ageing and ageing-related decline. Simple perception of the environment is sufficient to alter ageing and adult physiology in both nematode worms (Alcedo and Kenyon, 2004) and fruit flies (Libert *et al.*, 2007).

Evolutionary theory suggests that, during times of famine, diversion of resources away from reproduction towards somatic maintenance will increase the chances of an organism surviving to more plentiful times and thus increase long-term reproductive success (Kirkwood and Shanley, 2005). The selective advantage of shifting resources from reproduction to maintenance when food is restricted could be the 'public' factor shared between diverse organisms. It is as yet unclear whether these mechanisms are evolutionarily conserved across taxa or if instead this process is an example of convergent evolution.

1.5.3. Mating history

Connecting sex with lifespan is not a new concept because ageing is often accompanied by sexual decline. For hundreds of years, miracle cures have been purported to increase lifespan by reducing sexual indulgence (Wickens, 1998). In fruit flies, selection on late egg-laying capacity in females results in populations with extended lifespan but decreased early fecundity (Luckinbill *et al.*, 1984; Rose, 1984). Furthermore, long-lived populations resulting from selection for increased lifespan showed reduced early fecundity (Zwaan, 1995) and abolishing reproduction either through irradiation or genetic manipulation removes the differences in ageing rate between controls and long-lived selected lines (Sgro and Partridge, 1999). Although, other experiments have shown that long-lived flies lay more eggs over the course of their lifespan (Leroi *et al.*, 1994) reduced fecundity does not necessarily lead to increased longevity (Mair *et al.*, 2004).

Reproductive status has a major effect on lifespan, particularly in female *Drosophila*. Virgin females live up to twice as long as mated cohorts (Helfand and Rogina, 2003). The mechanism behind this extended lifespan is a combination of the reduction in energy costs from lowered or delayed egg production, and the costs of mating itself. The cost of mating includes factors other than wear and tear (Chapman *et al.*, 1993). For example, the seminal fluid transferred by males to females during mating has a direct negative effect on female lifespan (Chapman *et al.*, 1995), and sex peptides in the seminal fluid, likely intended to improve sperm utilization and decrease re-mating frequency, also have negative repercussions (Wigby and Chapman, 2005). Males are usually shorter-lived than females, but virgin males are longer-lived than mated males and females (Partridge and Farquhar, 1981), suggesting male courtship also comes at a cost (Cordts and Partridge, 1996) and this cost shortens male lifespan irreversibly (Prowse and Partridge, 1997).

1.5.4. Ambient temperature

Drosophila lifespan can be extended by reducing the ambient temperature that it is maintained in and at 18^{o}C, lifespan is doubled and the presence of ageing biomarkers is reduced (Loeb and Northrop, 1917; Miquel *et al.*, 1976). Evidence suggests that the ambient temperature has a direct effect on metabolic rates and that this is inversely related to lifespan (Pearl, 1928; Fleming and Miquel, 1983). Interestingly, lowering the ambient temperature even more can induce a state of reproductive diapause in young flies (Tatar and Yin, 2001), where females arrest their egg development, and both males and females can survive in this state for about 3 months, and upon temperature elevation become reproductively active and have a normal lifespan.

1.5.5. Stress shock

Mild or non-lethal stress often has the paradoxical effect of benefitting the organism by increasing either longevity or resistance to a stronger stress; this phenomenon is known as hormesis (Minois, 2000). In *Drosophila*, short-term exposures to hot or cold temperatures lead to higher survival rates when exposed to more extreme temperatures later on in life (Chen and Walker, 1993; Krebs and Loeschcke, 1994). However, many

organisms boast greater levels of thermotolerance when dealt with any form of mild stress. Flies living for one or two weeks in hypergravity (levels greater than 1*g*, terrestrial gravity) at the beginning of their imaginal life (early development) are more resistant to heat than non-exposed flies (Le Bourg and Minois, 1997), and flies exposed to low doses of irradiation have increased survival when later exposed at 35^{o}c in dry air (Lamb and McDonald, 1973).

It is believed that increased exposure to stress increases survival in organisms because it stimulates various repair and maintenance pathways, which include enhanced DNA repair, DNA methylation, anti-oxidative enzymes and those that remove damaged macromolecules (Rattan, 2008). As a result, hormesis has been proposed to explain the life-extending benefits of DR, where lowered nutritional availability is regarded as a form of mild stress. Furthermore, an increase in heat shock proteins, glucocorticoid steroid stress hormones, proteasome activity and lysosomal autophagy have been recorded in rodents subjected to DR (Masoro, 2007).

A related mechanism by which mild stresses increase longevity involves the disposable soma theory (Kirkwood, 2002), where the organism allocates more energy to survival and maintaining the soma during the short period of time under duress. Thus, the condition of the animal is in a better condition once the stress has been removed. Long-lived mutants, such as insulin/ insulin-like signalling (IIS) mutants are often found to be resistant to many forms of stress (Giannakou and Partridge, 2007) and they have been found to regulate their metabolism in order to better deal with their lowered IIS (Minois, 2000), which may be perceived as lowered nutrient sensing.

1.6. Genetic interventions that extend lifespan

1.6.1. Introduction

Genes are known to play a substantial role in longevity and the process of ageing (Finch, 1990). The first major study of the heritability of longevity was in 1899, a year before Gregor Mendel's work on genes was uncovered. This study found a correlation between long-lived parents and long-lived offspring (Beeton and Pearson, 1899). However it should be noted that rich families were generally longer-lived and poor

families were shorter-lived during that era, and the inheritance of wealth was just as important as the inheritance of genes. However, other studies have since been proposed to support the theory that ageing is a heritable trait (Gudmundsson *et al.*, 2000; Heijmans *et al.*, 2000; Garinis *et al.*, 2008).

Knowledge that a major element of the ageing process is hereditary or under genetic control, however, does not necessarily mean that ageing is determined by a fixed genetic pathway. Two distinctly different means by which genetic elements could determine longevity can be imagined. One is that genetic elements could be providing the instructive and driving forces that result in the stereotypic changes we see as ageing. During development, a similarly complex biological phenomenon, genetic elements do provide the instructive and driving forces for the characteristic changes that take place in developing from a fertilized egg to a mature individual. Given this precedence it is possible that genetic elements may also play a substantial role in driving some aspects of the stereotypic changes seen during the ageing process.

In contrast, the genetic contribution to ageing could be limited to providing a predisposition for the organism to respond to changes during life. In this case genetic elements are not controlling the process but providing, in a passive manner, the limitations an organism may possess for responding to change or other challenges life requires. For example, if it were to be imagined that ageing was due to the accumulation of damage from toxic elements, such as oxidative damage to macromolecules, the ability to defend against these degenerative changes could determine the length and rate of ageing. By helping to set the amount of protective anti-toxic systems through a genetic endowment, genetic elements could determine lifespan and the rate of ageing without directly setting the course of the ageing process itself.

In practice, researchers have particularly focussed on mutations and interventions that lengthen lifespan, reasoning that interventions reducing lifespan may not be directly linked to the ageing process. With this approach in mind, genetic analyses using model organisms have robustly identified genetic and physiological pathways involved in longevity regulation. These gene systems are highly conserved ‘public’ mechanisms (Partridge and Gems, 2002) and their characterisation required the integration of data from yeast, worm, fly and rodents. Processes thought to be involved in metabolic control,

stress resistance, genetic stability and reproduction have been implicated to be important to longevity and I briefly describe the processes relevant to this thesis.

1.6.2. Genetics of ageing in *S. cerevisiae*

In *S. cerevisiae*, replicative ageing is measured by the number of daughter cells that a single mother cell produces during its lifetime. Newly formed daughter cells have their ability to replicate fully restored. However, mother cells that reach a certain age produce daughter cells that also have reduced replicative ability, implying the existence of a senescence factor(s) transferred in the cytoplasm during division (Kennedy *et al.*, 1994). This factor may have resulted from the expression of specific genes in late-life, leading to experiments searching for yeast genes that are preferentially expressed at different ages (Egilmez *et al.*, 1989). One of these genes, longevity assurance gene 1 (*LAG1*) has been found to be predominantly expressed in young cells and gradually declines in its expression with each generation. The gene codes for a protein involved in transporting glycosyl-phosphatidylinositol-anchored proteins across the cell membrane (Barz and Walter, 1999). A *LAG1* null mutation has no observable effect on cell growth or metabolism, but has a dramatic effect on lifespan, increasing the number of cell divisions by approximately 50% (D'Mello N *et al.*, 1994). One proposal is that the role of *LAG1* is to set a critical expression threshold, and if expression falls below it, is responsible for triggering ageing and cell death. This threshold is based on its original levels of expression in young cells and removing it before a threshold is set causes the yeast cell to live longer (D'Mello N *et al.*, 1994).

One set of genes, *RAS1* and *RAS2*, yeast homologues of the mammalian pronto-oncogene c-*ras*, have also been found to regulate cell metabolism and lifespan (Jazwinski, 1993). High levels of glucose in the environment can activate *RAS1* and the yeast cAMP protein kinase A (PKA) pathway leading to increased growth and shorter lifespan, while low levels of glucose result in increased lifespan and stress resistance. Thus, under-expression of *RAS1* and over-expression of *RAS2* in yeast cells have been found to increase longevity and stress resistance (Sun *et al.*, 1994; Longo *et al.*, 1996) (**Figure 1.6.1.**). This suggests that the opposing RAS signalling genes regulate the homeostatic response to the fluctuating environment of the yeast cell, and these signals are

responsible for longevity and stress resistance (Jazwinski, 2000). Such a process may therefore play a role in the mechanistic response to dietary restriction (DR) in yeast. Indeed, *hexokinase*, a similar gene involved in the detection and utilisation of glucose by the cell has also been found to regulate longevity. Thus, deletion of *hexokinase* is equivalent to a form of DR, where the cell is tricked into perceiving reduced nutrient availability and triggering life extending mechanisms (Lin *et al.*, 2000).

In yeast, DR is achieved by reducing the glucose concentration of the medium from 2% to 0.5%. This intervention has been found to robustly extend yeast replicative lifespan (Jiang *et al.*, 2000). Recent work has suggested that DR exerts its effects by activating *Silent mating type Information Regulator 2* (*SIR2*). SIR2 belongs to a highly conserved class of proteins known as sirtuins, which function as NAD dependent deacetylases or ADP ribosylases (Lin *et al.*, 2000), altering the expression of relevant genes by removing acetyl groups from histone proteins in response to environmental conditions (Longo and Kennedy, 2006). Reduction of the activity of SIR2 has been found to extend chronological lifespan during poor nutrient conditions or when its nutrient sensing capabilities are affected by mutations in *RAS2* or *SCH9* (Fabrizio *et al.*, 2005). Another method by which SIR2 could extend lifespan is by inhibiting the recombination of ribosomal DNA (rDNA), which suppresses the accumulation of extra rDNA circles (ERCs) and thus extends yeast replicative lifespan by 40% (Kaeberlein *et al.*, 1999). Although increased activity of *SIR2* orthologues is associated with lifespan extension in worms and flies (Tissenbaum and Guarente, 2001; Rogina and Helfand, 2004), there is no evidence of a link between ERCs and ageing in any organism other than yeast.

The molecular and physiological bases of the effects of SIR2 and its role in DR are controversial with conflicting data about its role in the literature. For example, certain genetic stocks that lack a functional *SIR2* gene show little or no increase in longevity when subjected to DR (Lin *et al.*, 2000), supporting the view that the lifespan response to DR in yeast cells is dependent on SIR2. While several other *SIR2* null strains of yeast show a normal increase in lifespan in response to lowered glucose concentration in the food medium (Jiang *et al.*, 2002; Kaeberlein *et al.*, 2004) and the only yeast strain in which lifespan extension by glucose restriction is shown to require functional SIR2 does not show increased longevity when it is over-expressed (Kaeberlein *et al.*, 2004). Conversely,

in other long-lived yeast strains, over-expression of *SIR2* does extend lifespan but it is not required for increased replicative lifespan by DR (Kaeberlein *et al.*, 2004).

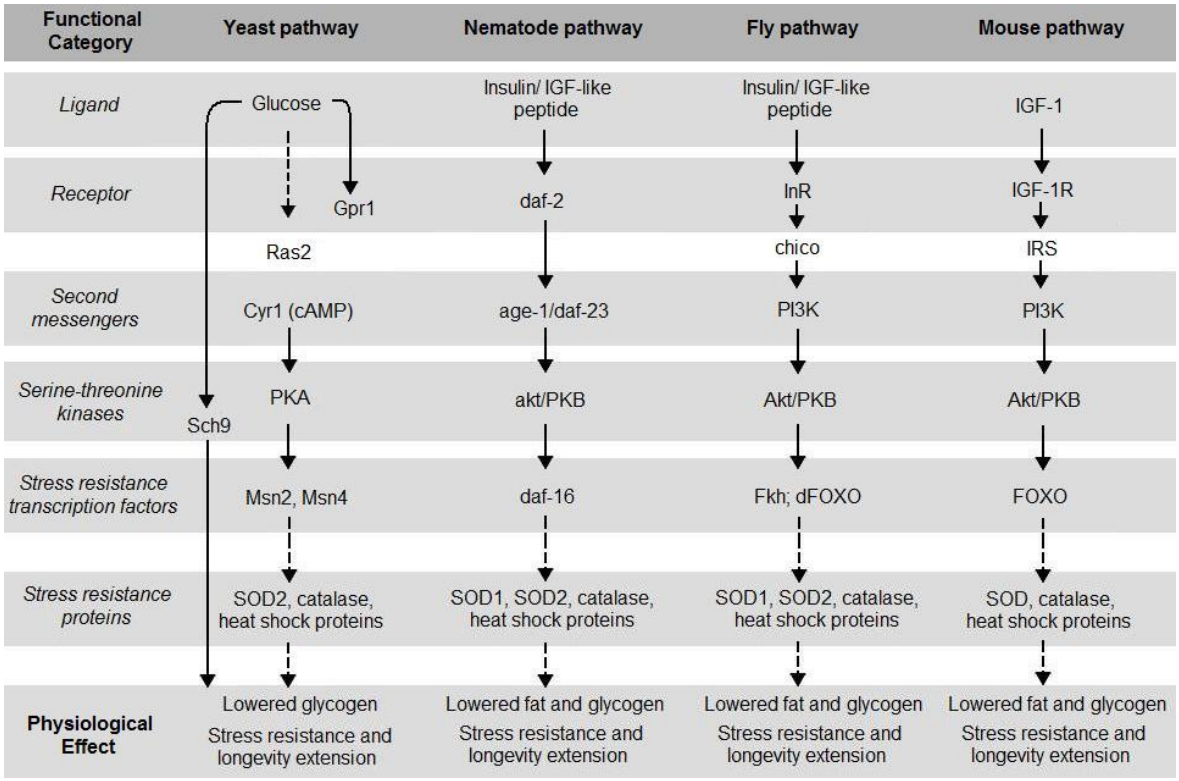

Figure 1.6.1 The pathways found in the four model organisms perform similar functions.
The yeast dietary restriction/stress-response pathway is compared here with the insulin/ insulin-like signalling (IIS) pathway of the nematode, fly and mouse. These functional similarities arise out of their common evolutionary origin. Dashed lines indicate unknown intermediates or unknown mechanisms of regulation.

1.6.3. Genetics of ageing in *C. elegans*

The first descriptive analysis of whether single genes could affect longevity was performed by Michael Klass (Klass, 1977), and throughout the 1980s, long-lived worms were generated, isolated and examined for survival. Some of the worms constitutively formed dauer larvae, a response specific to nematodes where worms are developmentally arrested to survive harsh conditions, while others were found to have a reduced feeding phenotype (Klass, 1983). These isolated strains were believed to be long-lived either because they were restricting their own nutrition or was somehow triggering the nematode dauer response in adults. One mutation, *age-1*, identified as the phosphatidylinositol-3-hydroxyl kinase (PI3K) in the worm version of the insulin/ insulin-

like signalling (IIS) pathway, was measured to be 30-60% longer-lived than the wild-type strain (Friedman and Johnson, 1988; Morris *et al.*, 1996). Further genes upstream and downstream of this pathway were later found to effect longevity when manipulated (Dorman *et al.*, 1995), which includes *daf-2* (Kenyon *et al.*, 1993), the orthologue receptor upstream of the IIS pathway, IGF-1R (Kimura *et al.*, 1997); and *daf-16*, the orthologue of the Forkhead bOX, sub-group O (FOXO) transcription factor target (Lin *et al.*, 1997; Ogg *et al.*, 1997), downstream of the IIS pathway. The IIS pathway has since been found to be evolutionary conserved in yeast, *Drosophila* and mammals (**Figure 1.6.1**).

The sensory perception of the abundance of food (bacteria in the case of the nematode) is related to the role of IIS in ageing, because neuron-specific promoters driving *daf-2* was sufficient in restoring normal wild-type lifespan in *daf-2* mutants (Wolkow *et al.*, 2000). Environmental signals are received by the sensory cilia and transduced to the neurons, which if the perceived conditions are right, produce an insulin-like protein ligand (Apfeld and Kenyon, 1999). This ligand binds to the DAF-2 receptor kinase in the membrane of the somatic cell and activates a signalling cascade resulting in DAF-16 phosphorylation (Riddle *et al.*, 1981; Vowels and Thomas, 1992). Unphosphorylated DAF-16 represses genes needed for reproductive growth and metabolism in the nucleus, while phosphorylated DAF-16 leaves the nucleus and can no longer act as a repressor. This leads to the development of a reproductive adult with a normal lifespan (Gottlieb and Ruvkun, 1994). This model suggests that lifespan mediation by the IIS pathway like in yeast involves shifting the organism between two functional states. One for longevity when the environmental conditions are adverse, such as overcrowding or low nutrition, and one for growth and reproduction, when environmental conditions are benign. However, reduction of function in *daf-2* by RNA interference during development only affects adult fecundity, whereas reducing function during adulthood affects only lifespan (Dillin *et al.*, 2002), suggesting that the effects on fecundity and lifespan are independent.

Another set of mutations identified to extend longevity in worms are the *clock* (*clk*) genes. These are a collection of at least four different genes which slow down by at least two-fold the growth, behaviour, development, and physiology of the nematode, consequently extending lifespan by about 30% (Lakowski and Hekimi, 1996). However, there is an additive effect on lifespan with mutants progressively longer lived with each

additional *clk* mutation, which suggests the *clk* genes do not affect lifespan via the same pathway (Lakowski and Hekimi, 1996). For example, the *clk-2* mutation extends lifespan by either altering gene expression or protecting the DNA, via regulating the length of telomeres (Benard and Hekimi, 2002). Whereas the *clk-1* gene encodes the mitochondrial enzyme that makes ubiquinone (coenzyme Q), an important co-factor during oxidative phosphorylation (Hekimi, 2000). Many isoforms of Q exist but *clk-1* mutants do not produce the most common endogenous form, Q_9 (Miyadera *et al.*, 2001). Wild-type and *daf-2* worms fed on bacteria that do not produce Q_8 are 50-60% longer lived (Larsen and Clarke, 2002) but *clk-1* mutants fed on a Q_8 deficient diet die during development, suggesting *clk-1* and *daf-2* extend lifespan via different and independent pathways. These pathways may involve reactive oxygen species (ROS) production, and it has been reported that Q_9 mutants produce 30-50% less ROS and therefore suffer less oxidative damage. This may explain how *daf-2: clk-1* double mutants are up to five times longer lived than wild-type worms because they reduce ROS through different methods (Lakowski and Hekimi, 1996).

1.6.4. Genetics of ageing in *D. melanogaster*

Insulin/ insulin-like growth factor signalling (IIS) is a highly conserved pathway found in many model organisms and orthologues of genes responsible for lifespan extension in nematodes have a similar effect in the fruit fly (**Figure 1.6.1**) (Giannakou and Partridge, 2007). Ablation of the cells which produce the ligands (*Drosophila* insulin-like peptides, DILPs) for this pathway have been documented to extend lifespan, reduce fecundity and increase stress in flies (Broughton *et al.*, 2005). Whereas, heteroallelic homozygotes of two different mutations in the insulin receptor gene (*InR*, the equivalent of *daf-2* in the worm) give rise to sterile dwarves with approximately 85% extended mean lifespan and 45% extended maximum lifespan (Tatar *et al.*, 2001). Mutation of the insulin receptor substrate gene, *chico*, also produces long-lived sterile dwarves (Clancy *et al.*, 2001) which suggests that down-regulation of the InR and/ or its substrate reduces IIS activity sufficiently to targets such as *Drosophila* Forkhead bOX, sub group O (dFOXO), a downstream transcription factor important for regulating cellular processes and lifespan

(Giannakou *et al.*, 2004; Hwangbo *et al.*, 2004; Carter and Brunet, 2007). The *dFOXO* gene is shown to have sequence homology with *daf-16* (Kramer *et al.*, 2003), and like in the worm, normal function of *dFOXO* is required for *InR* mutant longevity (Hwangbo *et al.*, 2004). Furthermore, over-expression of *dFOXO* in gut and abdominal fat-body is sufficient to extend adult lifespan without affecting fecundity or body size (Giannakou *et al.*, 2004; Hwangbo *et al.*, 2004; Giannakou *et al.*, 2007), suggesting that lifespan and fecundity effects are independently mediated by IIS (**Figure 1.6.2**).

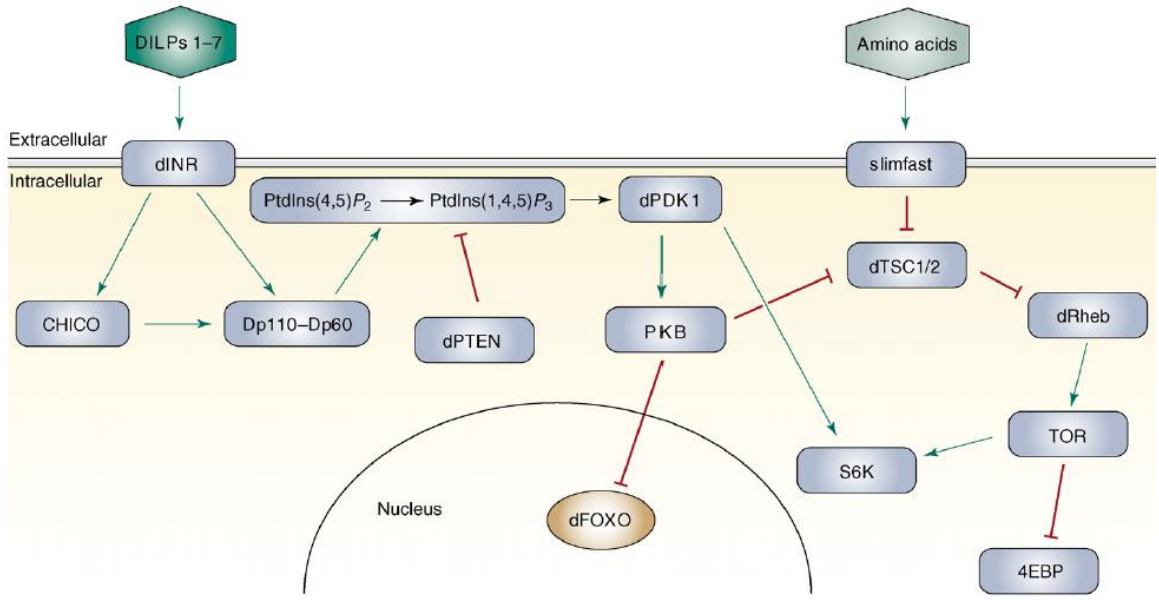

Figure 1.6.2 The known major components of *Drosophila* IIS pathway.
Drosophila has single genes encoding all components of the IIS pathway apart from the DILPs, where there are seven genes encoding seven different insulin-like molecules. In the *Drosophila* adult, dFOXO transcriptional activity is inhibited by IIS, and therefore reduction of IIS flux by disrupting one of its components leads dFOXO mediated lifespan extension. Figure reproduced from (Giannakou and Partridge, 2007).

Drosophila Target Of Rapamycin (dTOR) signalling, like IIS, is also a nutrient sensing pathway that is involved in the regulation of growth, size, ageing and autophagy, a process that degrades the cell's own components through the lysosomal machinery (Klionsky, 2004). The role of dTOR in ageing involves sensing amino acid availability to modulate activity of the S6 kinase (S6K) regulatory gene to enhance growth and repress longevity. Decreased dTOR activity by either dominant-negative mutations in *dTOR* and *S6K*, or by over-expression of upstream inhibitors of dTOR, tuberous sclerosis complex 1 or 2 (dTSC1 or dTSC2), leads to extended longevity in fruit flies (Kapahi *et al.*, 2004). These

discoveries have demonstrated that *Drosophila* lifespan can be manipulated by single gene manipulations and the pathways involved are inherently linked with growth, reproduction and stress resistance.

1.6.5. Genetics of ageing in *M. musculus*

Like invertebrates, mammals such as mice also have an IIS pathway which when disrupted cause defects to growth but may also extend lifespan (**Figure 1.6.1.**). Growth hormone (GH) is delivered into the blood stream from the anterior pituitary gland and when GH receptors are bound, they activate synthesis and secretion of insulin growth factor-1 (IGF-1), the ligand of the mammalian IIS pathway (reviewed in Berryman *et al.*, 2008). Snell and Ames mice have homozygous mutations which inhibit the development of pituitary cells, hindering the production of GH and thus lowering the level of IGF-1 signalling. Despite the consequent dwarfish appearance, these mice models are long-lived and have reduced biomarkers of ageing such as reduced tumour development and delayed decline in both memory and locomotor activity (Liang *et al.*, 2003). Further studies with over-expression of *Klotho* (a suppressor of IGF-1 signalling) (Kuro-o, 2008), heterozygous null mutants of the IGF-1 receptor (Holzenberger *et al.*, 2003) and IRS1 deletion mutants (Selman *et al.*, 2008) are long-lived but do not develop dwarfism, and have unaffected physical activity, fertility and reproduction. However, a common trait shared among these long-lived models is decreased IGF-1 activity. Thus, the similarities in the insulin/IGF-1 and homologous regulatory systems between invertebrates and mammal models with increased longevity suggest that a fundamental mechanism of ageing is likely to be evolutionarily conserved.

1.6.6 Have pathways evolved as an adaptation to environmental signals?

How can the existence of conserved pathways that regulate the rate of ageing be reconciled with the viewpoint that ageing exists because of selection's indifference (**1.3**)? One possibility is that some of these pathways allow organisms to resist ageing by altering their allocation of resources in response to environmental signals. Evolutionary biologists have long recognized the powerful influence that environmental cues have on biological

systems, an influence that can trigger alterations in life history patterns that range from the subtle, such as delayed reproduction and increased stress resistance, to the dramatic, such as abrogation of normal development entirely and permanent acceptance of a juvenile form. From an evolutionary standpoint, the benefit of such plasticity is clear. Variable environmental conditions challenge individuals to use external information and make calculated decisions about whether to allocate resources to somatic maintenance (i.e., short-term survival) or to reproduction to maximise their individual fitness. In this light, the existence of molecular mechanisms to modulate or enact such decisions is not surprising. The existence of conserved regulatory pathways may explain why some mutations have dramatic effects on lifespan. While there are indeed many genes with small effects on ageing (Murphy *et al.*, 2003), these genes are subject to regulation at a high level. Mutations that impact regulation may lead to the more dramatic effects on ageing that were so surprising from an evolutionary standpoint.

The experimental data above suggests that lifespan is responsive to alterations to genes involved in either stress response or nutrient sensing. While benign conditions might favour organisms that reproduce quickly and invest little in maintenance and stress resistance, stressful conditions might favour organisms that invest strongly in maintenance and delay reproduction until conditions improve. Environments that vary between benign and stressful can thus select for organisms that can sense the state of the environment and change their life-history strategy accordingly. Therefore, two general processes may have evolved, one for increased nutrition (growth and reproduction) and another for stress response (survival). When we manipulate the genes which are involved in these two processes, we are observing the effect of switching the organism between the benign and harsh environmental conditions.

1.7. Thesis Outline

The vast majority of interventions that extend lifespan were first discovered in model organisms such as rodents and the nematode worm. But before any lifespan-extending interventions can be applied to humans, it must be shown that these manipulations are not a feature unique to one particular species and they robustly extend lifespan

substantially and healthily across all taxa. There is therefore a clear benefit to elucidate these processes in lower organisms first. Indeed as described above, the IIS pathway is involved in determining lifespan as well as size and growth across a broad range of model organisms and DR has been shown to be effective in extending lifespan through decreased nutrient intake in a wide range of organisms. However, the mechanisms underlying these processes are yet to be fully characterised or understood.

Furthermore, confusion and gaps in our knowledge regarding both processes still exist. IIS mutants like *chico* have robustly increased lifespan (Clancy *et al.*, 2001), but the exact method of how CHICO signals lifespan-extending downstream components like dFOXO is unclear because disruption of components in between are either non-viable (Stocker *et al.*, 2002) or not long-lived (Clancy *et al.*, 2001). And amongst the useful information arising from DR studies in *Drosophila* (Mair *et al.*, 2005) conflicting results have been reported as to whether DR causes lifespan extension in flies at all (Le Bourg and Minois, 1996; Carey *et al.*, 2002; Carvalho *et al.*, 2005).

Therefore, the main aim of this thesis is to target some of the known issues in *Drosophila* ageing research. Much of the confusion may be a result of either a lack of fundamental knowledge required for *Drosophila* ageing studies or a lack of convention in between *Drosophila* labs, especially regarding DR implementation. In order to clarify and understand the effect of any intervention in *Drosophila*, experimental protocols must be standardised. Only once this has occurred can the mechanisms involved in lifespan-extension be fully evaluated. The following sections outline the experiments performed to (1) optimise *Drosophila* DR protocols, (2) test that *Drosophila* subjected to DR are nutritionally restricted, (3) test whether disruption of IIS lifespan extension is mediated via dAKT, a pathway between CHICO and dFOXO.

1.7.1. Do differences in laboratory practices affect the outcome of DR in *Drosophila*?

Different lifespan responses from DR experiments have been reported in *C. elegans* and rodents because of, respectively, the use of different bacterial strains as food (Garsin *et al.*, 2001) or by interchanging casein and soy peptone as the source of dietary protein (Iwasaki *et al.*, 1988). In *Drosophila*, yeast is the most important ingredient in the DR

lifespan response (Mair *et al.*, 2005) but little is understood about the mechanism involved. In general, as the nutrient concentration increases, lifespan increases to a peak and decreases even as nutrition continues to increase. If fecundity increases when lifespan decreases at high concentrations of nutrient availability, then evolutionary theory suggests somatic maintenance, thus lifespan, is traded-off to allow the animal to maximise its reproduction (Shanley and Kirkwood, 2000). But if a high concentration of nutrient availability decreases lifespan without an increase in fecundity, then the decrease in lifespan is likely to be a result of a toxin.

Currently, different fly laboratories studying DR use different sources of yeast and different concentrations of yeast, sugar and agar in experiments (Chippindale *et al.*, 1993; Chapman and Partridge, 1996; Kapahi *et al.*, 2004; Bross *et al.*, 2005). Despite these differences, few laboratories have tested their diets by measuring fecundity to ensure that the lifespan extension observed is a result of a trade-off rather than a toxin. Furthermore, these laboratories use different genetic backgrounds and differing techniques for long-term maintenance of their fly stocks, factors that add complexity and possible laboratory artefacts to DR studies. In chapter 3, the differences in yeast composition and differences in laboratory diets are compared for their affect on the fly DR response, and in chapter 4, the differences in genetic background on the fly DR response are analysed in greater detail.

1.7.2. Can we measure the level of nutritional intake in flies subjected to DR?

In *Drosophila*, dietary restriction is generally implemented by dilution of yeast in a sugar-agar based medium (Chapman and Partridge, 1996). However, flies subjected to DR are maintained on and have constant access to diluted food medium and it assumed that they receive less nutrition than control flies. However, flies could compensate for the reduced nutrient availability in the food by increasing their total feeding intake. A recent study that estimated *Drosophila* feeding rate by using radioactively-labelled food reported compensation for food dilution (Carvalho *et al.*, 2005), whereas another study has shown that experimental flies do not differ in their feeding (Mair *et al.*, 2005). The ability to measure food intake in flies is difficult because of their small size and unlike in rodents, flies consume volumes of food which are too low to weigh accurately. In chapter

5, I describe experiments designed to optimise a protocol that can accurately measure the total feeding intake in *Drosophila*, which is useful not just for DR and lifespan but also for drug uptake and nutrient sensing studies. This protocol was then used to assess *Drosophila* feeding in a range of conditions including DR.

1.7.3. The role of AKT signalling in *Drosophila* ageing

Lifespan extension has been reported in fruit flies with dFOXO over-expression in the adult fat body (Giannakou *et al.*, 2004; Hwangbo *et al.*, 2004). dFOXO is a transcription factor important for regulating cellular processes including growth and lifespan. The IIS pathway is known to phosphorylate dFOXO preventing it from activating longevity gene targets in the nucleus (Giannakou and Partridge, 2007) and therefore the IIS disruption by either ablation of the cells that produce the IIS ligands (Broughton *et al.*, 2005), loss of function mutations in the receptor of pathway (Tatar *et al.*, 2001), or null mutation of *chico* the receptor substrate (Clancy *et al.*, 2001) also extend fly lifespan. However, the interaction between CHICO and dFOXO in relation to lifespan is unclear.

Aside from lifespan extension, the IIS pathway is also an important regulator of growth and metabolism, and much of the literature regarding the interemediates between CHICO and dFOXO, such as dPI3K and dAKT, is focussed on the role they play in growth and development in pre-adults, and less on their role in adult lifespan. It would be of great interest to elucidate the role of dAkt in fly lifespan and experiments to achieve this are described in chapter 6.

Chapter 2: General methods and materials

2.1. *Drosophila melanogaster* stocks

2.1.1. Dahomey wild-type

The wild-type (wt) stock was collected in 1970 in the Republic of Dahomey (now called the Republic of Benin), West Africa and has been maintained in parallel since collection in four population cages (measuring 20 x 21 x 30cM) with overlapping generations on a 12-hour light/dark cycle in a 25^{o}C, non-humidified controlled temperature (CT) room. This method has been shown to maintain lifespan and fecundity at levels observed in freshly collected flies (Sgro and Partridge, 2000). Flies in population cages had constant access to 12 bottles of standard Sugar-Yeast (SY) food medium, with the three oldest bottles being replaced with fresh medium every week.

2.1.2. *white*^{1118} stock

The *white*^{1118} (3605), abbreviated to w^{1118}, mutant was originally obtained many years ago from the Bloomington *Drosophila* stock centre at Indiana University, and have been maintained at varying ages in bottles containing SY food medium at 18^{o}C in a humidified CT room.

2.1.3. *white*^{Dahomey} stock

Backcrossing w^{1118} flies into the Dahomey wt background derived the $w^{Dahomey}$ stock. These were then maintained at varying ages in bottles containing SY food medium at 18^{o}C in a humidified CT room.

2.1.4. General stock maintenance

Most large fly laboratories maintain stocks that are not in everyday use at 18^{o}C on a 4-5-week generation cycle. Stocks should be kept as two to four independent cultures, and it may be convenient to keep these on alternating generations, 2 weeks apart. Stocks

are normally maintained in bottles. Other than the Dahomey wt stock, all fly stocks were kept in several bottles containing SY food medium at 18^{o}C in a humidified CT room. Bottles contained overlapping generations of flies and this was maintained by transferring flies to a fresh set of bottles every 4-5 weeks.

2.2. *Drosophila* food medium

2.2.1. Laboratory sugar/yeast (SY) medium

In chapter 3, standard (1x) SY food contained per litre: 100g autolysed Baker's yeast (B.T.P. Drewitt, London, UK), 100g sucrose (Tate & Lyle sugars, London, UK), and 10g agar (Sigma-Aldrich, UK), distilled water to 1 litre. 30ml Nipagin M solution (100g/L methyl 4-hydroxybenzoate in 95% ethanol) (Clariant UK Ltd, Pontypridd, UK) and 3ml propionic acid (Sigma-Aldrich, UK) were also added as preservatives.

In chapters 4, 5 and 6, the standard (1x or 1.0) SY food contained per litre: 100g autolysed Brewer's yeast (MP Biomedicals, Solon, OH, USA), 50g sucrose (Tate & Lyle sugars, London, UK), 15g agar (Sigma-Aldrich, UK), distilled water to 1 litre. 30ml Nipagin M solution (100g/L methyl 4-hydroxybenzoate in 95% ethanol) (Clariant UK Ltd, Pontypridd, UK) and 3mL propionic acid (Sigma-Aldrich, UK) were also added as preservatives.

Foods used for DR experiments were prepared on a gas hob with the following protocol: the distilled water and agar were first brought to a rolling boil, at which point the sugar and yeast were stirred in to dissolve at a simmer. The mixture was allowed to cool to 60^{o}C at which point the nipagin and propionic acid (anti-fungal and anti-bacterial agents) were stirred in. The SY medium was then immediately dispensed into appropriate containers and allowed to cool and set.

Food used for stock maintenance was cooked in a 60 litre Joni Multimix food preparation kettle (Joni Foodline, Munkebo, Denmark), whereby the cooking process described above was electronically controlled. Foods were stored at 4^{o}C and kept for no longer than 3 weeks.

2.2.2. Alternative SY medium in DR experiments

Experiments that involved DR (chapters 3, 4 and 5) occasionally subjected flies to foods of varying nutritional quantity. This involved increasing or decreasing the number of grams of sugar and/ or yeast per litre of food while keeping all the other ingredients in the SY medium constant. In chapter 3, 10g of sugar and yeast added to 1 litre of food is denoted as 0.1SY food, 20g of sugar and yeast added is denoted as 0.2SY food, and so forth. In chapter 4 and 5, the concentration of sugar was fixed at 50g/L and only the yeast concentration varied.

2.2.3. Grape medium

Grape medium contained 50g agar, 600ml red grape juice, 100ml extra water and 42mL nipagin (100g/L) dissolved in 1 litre of distilled water. The water and agar was brought to the boil before adding the grape juice. This mixture was brought to the boil again, the extra water was added, and was allowed to cool down to 60^{o}C before the nipagin was added. The final content was poured into a plastic Petri plate and allowed to set.

2.3. General methods and animal husbandry

2.3.1. Separating males and females

Males and females are distinguishable due to differences in the appearance of their genitalia located at the base of the abdomen. Males are also generally smaller than females, possess ‘sex comb’ structures on their forelegs used in courtship and contain a characteristic dark pigmented area on the dorsal posterior section of the abdomen (Ashburner, 1989). Based on these differences in appearance the males and females can be separated using a fine paintbrush under CO_2 anaesthesia.

2.3.2. Virgin collection

Although some variation between stocks exists, the general rule is that females will not accept a male mate until 8-12 hours after eclosion from pupae (Ashburner, 1989). Thus, flies can be collected during this window (or, better, between 6 and 8h after eclosion), anaesthetized, separated into males and females, and stored until needed in yeasted vials. The females will then usually be virgin when used. As a preliminary check, the vials that were used for storing the virgin females should be kept and inspected 3 or 4 days later for any signs of larvae. If larvae are present, it is clear that at least one female in that vial was not virgin, and the vial should be discarded. The following is a convenient schedule for virgin collection:

Day 0: Discard all flies from emerging cultures in evening. Store emerging cultures at 18^{o}C overnight in the dark. *Day 1:* Discard all emerged flies from the cultures first thing in the morning. Put cultures at 25^{o}C in the light. About 5 hours later collect by CO_2 anaesthetisation all emerged flies. Separate into males and females, and store these in separate vials at 25^{o}C until required. Those that are relatively unpigmented and/ or have unexpanded wings will almost certainly be virgin. Return the emerging cultures to 25^{o}C in the light and possibly collect virgins last thing in the evening. Keep the 'female' vials and inspect 3-4 days later for larvae. If larvae are present, presume that any females from that vial were non-virgin. The presence of eggs in the female-holding vials is not evidence of non-virginity; even virgin females will lay eggs, albeit at a lower rate in comparison with mated females.

2.3.3. Standard larval density

To control the effects of parental age and larval density on longevity (Priest *et al*., 2002) both the parents of experimental flies and experimental flies were of the same age and reared at constant density. This was achieved by placing a grape plate into the population cage for flies to lay eggs on. Egg-laying on the plate was assisted with some live yeast smeared on the surface. After a 20-24h lay, the plate was removed and the eggs washed off into a 15mL Falcon tube using phosphate buffered saline (PBS) solution. Eggs were allowed to settle at the bottom before being dispensed into a 200mL bottle

containing 70mL SY medium using a Gilson pipette. Each dispersion contains 20μL of egg suspension, equating to 300 - 350 eggs per bottle.

2.3.4. Setting up crosses and backcrossing

Crosses were set up according to the needs of the experiment. For experiments, the parental generation were reared at standard larval density from which virgin females were collected. A minimum of fifty mating pairs were used in crosses, which occurred in yogurt pot cages containing a grape medium Petri plate with live yeast paste smeared on the surface. Flies were allowed to mate for 24h and eggs to be laid on the surface. Eggs were then dispensed to bottles to achieve standard larval density in the experimental generation. Experimental lines that were developmentally delayed for a few days were set up earlier to allow synchronised eclosion with controls.

For backcrossing mutants, the F1 generation was created by crossing mutant males to virgin females in the outbred background. This ensured the transfer of cytoplasmic constituents from the background to the progeny. Virgin females carrying the mutation were collected and crossed to males of the outbred background to generate the F2. This process was repeated for a minimum of 10 generations. Balancers were also backcrossed and mated with the mutant line at the end of the scheme.

2.3.5. Lifespan assays

All lifespan experiments were performed with 'once-mated' female *Drosophila*. Experimental flies were reared at standard larval density at 25^{o}C. The flies were transferred to new SY bottles following a 24h eclosion period and allowed to mate over a 48h period. Females were then collected under CO_2 anaesthesia and assorted in either bottles or vials. For lifespan assays that used 35mL vial containers, the starting density was 10 flies per vial, and each vial contained 5mL of SY food. For lifespan assays using 200mL bottle containers, the starting density was 100 flies per bottle, and each bottle contained 50mL of SY food.

Over the course of the assay the flies were kept in the 25^{o}C, 65% humidity CT room in a 12: 12 hour, light: dark cycle. Flies were transferred to fresh food every other day and

scored for deaths and censors. Flies were recorded as censored when found alive but immobilised on the food, or when flies escape during transfer.

2.3.6. Fecundity assays

Experimental flies were kept in the same glass vials for between 18 and 24h at which point they were transferred again to fresh food. The eggs in the vacated vials were counted by hand under a light microscope and the number of females per vial and the day on which they were laid were recorded. Generally, these counts were performed once a week for the first six to seven weeks of adult life. The index of lifetime fecundity is used to represent lifetime fecundity and is the sum of eggs laid during 24h on the days of counting by an average female.

2.4. Molecular biology

The majority of nucleic acid and protein manipulation techniques were performed in ice cold conditions. Flies were usually snap frozen in liquid nitrogen and stored at -80^{o}C until experimentation.

2.4.1. Genomic DNA extraction

Flies (*N* = 20) were ribolysed in 200µL of buffer containing 100mM Tris-HCl (pH7.5), 100mM EDTA, 100mM NaCl and 0.5% SDS. The homogenate was incubated at 65^{o}C for 30 min, followed by 10 min incubation on ice after being mixed with 800µL of LiCl/KAc solution (4.3 M LiCl and 1.43 M KAc). The mixture was centrifuged at 12,000RPM for 15 min at 25^{o}C. DNA was precipitated from the supernatant by addition of isopropanol and pelleted by centrifugation at 12,000RPM for 15 min at 25^{o}C. The DNA pellet was washed with 70% ethanol and dissolved in 50–150µL of TE (Tris-EDTA) buffer.

2.4.2. Polymerase Chain Reaction (PCR)

A PCR reaction typically contained 2.5µL (10x) PCR buffer, 1µL (10mM) dNTPs, 1µL genomic DNA, 1µL (6.6µM) each of a forward and reverse primer, 0.25µL TAQ polymerase, made up to 25µL with H_2O. Reagents were obtained from Qiagen, UK. PCR was performed using a thermal cycler (Eppendorf UK Limited), which for the majority of experiments was programmed to:

1) 94^{o}C for 15 minutes to denature the hot-start TAQ polymerase
2) 94^{o}C for 30 seconds to denature the DNA
3) 50-60^{o}C for 30 seconds to anneal the primers
4) 72^{o}C for 30-60 seconds to synthesise the DNA

   Steps 2-4 would be cycled for about 35 times
5) 72^{o}C for 10 minutes to allow additions of 'A' base tail for a uniform PCR product

2.4.3. Gel electrophoresis

1 – 2g of agarose was added to 100mL of TAE (Tris base, Acetic acid, EDTA) buffer in an Erlenmeyer flask and heated to the boil. This was cooled and 5µL of ethidium bromide (EtBr) was added and mixed. This was poured into a gel tray and a comb was added to create wells for the samples. This was allowed to cool and set. A marker ladder and samples containing 6x loading dye (40% glycerol, 6x TBE buffer and 0.25% bromo blue) was pipetted in 18-24µL volumes into the wells. The electrophoresis was performed between 70 – 100V until the marker reaches the bottom. After which the EtBr, which has intercalated in between the DNA, can be visualised under UV light.

2.4.4. RNA extraction

Whole bodies were homogenised for total RNA extraction, whereas head RNA was obtained by shaking fly bodies vigorously in an eppendorf after freezing in liquid nitrogen, and collecting the decapitated heads. Sampled tissue was homogenised in 1mL of Trizol (Gibco). After 5 minutes at room temperature (RT), 200µL chloroform was added and shaken vigorously. After 3 minutes at RT, the homogenate was centrifuged at 12,000RPM

for 15 minutes at 4^{o}C. The upper aqueous layer was removed to a new tube and RNA was precipitated in 500µL isopropyl alcohol and 50µL sodium acetate. After 40 minutes at -80^{o}C, the mixture was centrifuged and the pellet was isolated and washed in 70% ethanol.

2.4.5. Bradford assay

BioRad reagent (5x) (Bio-Rad protein assay reagent; Bio-Rad Laboratories (UK) Ltd, Hemel Hempstead) was added up to 900µL of H_2O. 1µL of protein sample was diluted in 99µL of H_2O and added to the Bradford reagent dilution. This was mixed and placed in a cuvette, and measured in a spectrometer at 595nM. Protein concentrations were determined using known volumes of bovine serum albumin (BSA) as a standard.

2.4.6. Sodium Dodecyl Sulfate PolyAcrylamide Gel Electrophoresis (SDS-PAGE)

3.75% stacking gel and 8% resolving gels was made for SDS-PAGE.

For 5mL of stacking gel mixture: 469µL 40% 37.5: 1 acrylamide/ bisacrylamide (A7168 Sigma-Aldrich, UK), 625µL 1M Tris-HCL (pH6.8), 50µL 10% SDS, 3.8mL of H_2O. To initiate polymerisation of the gel, 50µL ammonium persulphate (100 mg/mL) and 3µL of TEMED was added.

For 10mL standard resolving gel mixture: 2mL 40% 37.5: 1 acrylamide/ bisacrylamide (A7168 Sigma-Aldrich, UK), 1.25mL 3M Tris-HCL (pH8.8), 100µL 10% SDS, 6.53mL of H_2O. To initiate polymerisation of the gel, 100µL ammonium persulphate (100 mg/mL) and 20µL of TEMED was added.

For immunoblotting phosphorylated FOXO protein, the 8% resolving gel mixture contained: 112: 1 acrylamide/bisacrylamide rather 37.5: 1 acrylamide/bisacrylamide.

SDS-PAGE was performed using Bio-Rad Mini-PROTEAN 3 Cell and set-up according to manufacturer's instructions. Protein samples were buffered in 150mM Tris pH9.0, 4% SDS, 30% glycerol, 100mM DTT and 0.01% bromophenol blue. Samples were heated to 95^{o}C for 5 minutes before loading. As a marker for molecular weight Rainbow™ marker (Amersham plc) was loaded in an adjacent well.

2.4.7. Western blotting

Protein was transferred from the SDS-PAGE gel to Hybond-P™ membrane (Amersham plc) using Bio-Rad semi-dry electrophoretic transfer cell. The protocol was performed according manufacturer's instructions. Membranes were washed in Tris-Buffered Saline solution with Tween 20 (TBS-T), and blocked using solution containing skimmed milk powder (5% SMP) in TBS-T for 1hr at room temperature. After washing with TBS-T, the membrane was exposed to primary antibody (added with TBS-T and 5% Bovine Serum Albumin) overnight at 4^{o}C. After washing with TBS-T, the membrane was exposed to secondary antibody (added with TBS-T and 5% SMP) for 1h at room temperature, and then washed with TBS-T. Visualisation of conjugated anti-horseradish peroxidase secondary antibodies was performed using an electrogenerated chemiluminescence detection reagent and hyperfilm kit (Amersham plc) in a dark room.

2.5. Statistical analyses

Statistical analyses were performed using JMP 5.1 statistical software (SAS Institute Inc.).

2.5.1. Survival experiments

Survival curves were generated by the Kaplan-Meier estimation (Kaplan and Meier, 1958). Survival curves represent the proportion of individuals surviving at independent time points that arise from recording times of death of individuals in a population. The Kaplan-Meier estimation allows for the inclusion of the exact time of death or censorship of all individuals who were enrolled at any time during an experiment. Differences in survival between experimental cohorts were compared throughout using the non-parametric log-rank test (Miller, 1981) where the test statistic is chi-squared (χ^2) and $P \leq 0.05$ indicates the minimal significant difference between groups.

2.5.2 Fecundity experiments

The Wilcoxon test was used for analysis on differences of daily fecundity between conditions and differences of lifetime fecundity between conditions.

Chapter 3: Dietary restriction of *Drosophila* on differing yeasts

Abstract

Dietary restriction (DR) describes a moderate reduction of food intake that extends lifespan in a wide range of organisms, including the fruit fly Drosophila melanogaster. *However, the mechanisms underlying this phenomenon are currently unknown. One approach taken to identify these mechanisms is to determine if reduction of specific dietary components can extend lifespan in a manner similar to whole food reduction. However, since different laboratories often use different diets and techniques for implementing DR, the information from such investigations may not be strictly comparable. For instance in* D. melanogaster*, an important model for the study of DR, the nutritional content of diets is typically poorly defined, and few laboratories have adequately tested their diets to determine if they are appropriate for DR experiments. In the work reported in this chapter, a variety of fly diets composed of different sources were compared for their effect on fly lifespan and fecundity. Only one type of Brewer's yeast, at one sucrose concentration and over a narrow range of agar concentrations, was appropriate for DR experiments with flies. Additionally, DR by intermittent feeding was tested and found to have no effect on fly lifespan. Concluding that food dilution is the most practical and effective method of DR implementation and the optimised diet described in this chapter is the recommended method used in investigations of DR in* D. melanogaster*. These findings have been published in Bass* et al. *(2007)(see Appendix 1).*

3.1. Introduction

Dietary restriction (DR) refers to a reduction of food intake without malnutrition which results in increased survival. This intervention has principally been studied in rodents, but has also been found to extend the lifespan of a wide range of organisms including the fruit fly *Drosophila melanogaster* (Weindruch and Walford, 1988; Partridge *et al*., 2005b). A lengthy and well documented history of DR in rodents exists (Sacher and Thaler, 1977;

Yu *et al*., 1985; Masoro, 1988; Masoro *et al*., 1992; McCarter and Palmer, 1992) with the effect of DR first documented in rats (McCay *et al*., 1935). Two methods of implementing DR in rats and mice are in common use. In the first, a control group are fed *ad libitum* (AL) with the amount eaten recorded, the experimental DR group are consequently fed a reduced amount of that eaten by AL fed animals. For instance, male rats subjected to DR were fed 60% of that eaten by the AL group, which resulted in a 47% increase in median lifespan of the DR individuals (Yu *et al*., 1982). Such a method is often referred to as limited daily feeding (LDF). LDF animals are long-lived, often have lowered body weight, lowered stores of energy metabolites but are less prone to diseases such as cancer (McCay *et al*., 1935; Masoro *et al*., 1980; Yu *et al*., 1982; Yu *et al*., 1985).

In the second method, food is provided AL to both the control and DR groups, but the experimental group is subjected to periods of intermittent feeding, this method maybe referred to as either intermittent fasting or intermittent feeding (IF, in both cases). In recent studies, a regime is in place where food is provided to DR cohorts every other day (EOD) (Goodrick *et al*., 1990; Anson *et al*., 2003). In these experiments, the EOD cohort has alternating bouts of 24h AL access to food followed by 24h of starvation, while controls have continuous AL access to food. However, DR mice were found to gorge themselves when provided with unlimited food after 24h of starvation, and the overall weekly caloric intake and body weight of DR mice were similar to that of control groups (Anson *et al*., 2003). Despite compensating for periods of fasting, EOD fed mice exhibit extended lifespan and reduced risk of cancer, suggesting the beneficial effects of DR are not the result of decreased calorie intake (Anson *et al*., 2005).

In DR studies with rats, the laboratory diet offered is semi-synthetic which consists of casein as the protein source, corn oil as the fat source, dextrin and sucrose as the carbohydrate source. The LDF diet is supplemented so that all animals have similar intakes of vitamins (Yu *et al*., 1982; Yu *et al*., 1985). Due to the defined and well controlled list of ingredients in this diet, the source of dietary protein has been discovered to be important, with rat lifespan further extended when casein is replaced by soy peptone (Iwasaki *et al*., 1988). Further studies have also revealed that specific reduction of single amino acids, such as methionine, can extend lifespan in rats (Orentreich *et al*., 1993; Zimmerman *et al*., 2003) and mice (Miller *et al*., 2005). Restriction of the amino acid, tryptophan, has also been implicated to increase lifespan to

the same extent that whole food restriction would in rats (Segall and Timiras, 1976; Ooka *et al*., 1988) and in mice (De Marte and Enesco, 1986). In some cases, the effects of protein adjustment are smaller than those of whole food DR, which has sparked debate about whether all dietary reductions operate through the same mechanism(s) to extend life.

In comparison to rodents, the response to DR in *D. melanogaster* is less well characterised. There are three methods of restricting nutrition in the fruit fly: by IF (Kopec, 1928), by dilution of live yeast (Alpatov, 1930) and by dilution of a yeast-based medium (Chapman and Partridge, 1996).

DR by IF is similar to the way IF is implemented in rodents. Flies are allowed to feed AL for a fraction of the day on live yeast and the length of the fraction is varied accordingly. A decrease in lifespan was found in flies that were only allowed to feed for short periods of time, but a generally positive effect on lifespan was observed when flies were subjected to longer periods of feeding (18h access to food and 6h access to water only in every 24h) (Kopec, 1928). However, the low lifespan of all experimental cohorts, which may indicate flies were otherwise malnourished, coupled with the lack of statistics suggest the results are inconclusive. Furthermore, a more recent study has reported no positive effects of this treatment or of any other treatments in which the timing of the starvation/ feeding periods was altered (Le Bourg and Medioni, 1991). However, in this latter study, the treatment was only implemented on 5 out of every 7 days of adult life, making it possible that any beneficial effects of the protocol were masked by the days without treatment. The results from IF could indicate 1) imposing DR by intermittently feeding and intermittently starving flies does not work 2) flies cannot be dietarily restricted. Thus, it would be of great interest to test which conclusion is correct.

DR by dilution of live yeast involves flies having free access to varying quantities of a limited amount of live yeast added to a base medium usually containing (but not always) corn flour, sucrose, dead yeast and agar (Alpatov, 1930). This procedure has yielded conflicting reports with either a 25-30% lifespan extension observed in females (Chippindale *et al*., 1993) or no lifespan extension found in females (Le Bourg and Minois, 1996). Such differences may stem from variations in experimental procedures such as the use of a different wild-type strain of *Drosophila* and/ or the use of a different food base

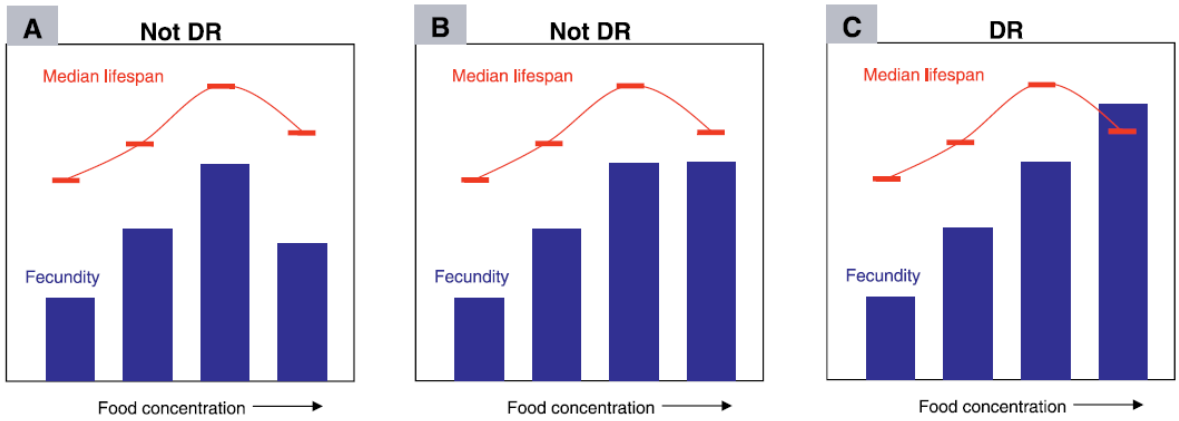

Figure 3.1.1 The responses of lifespan and fecundity to food concentration that is required for DR studies in *Drosophila*.
As food concentration increases from starvation, lifespan should increase to a peak at DR, from which it declines due to a nutrient-dependent effect of 'high' food. If fecundity decreases (A) or is unchanged (B) by the nutrient increase that decreases lifespan, the most likely explanation for shortened lifespan is toxicity. To minimise the possibility that food toxicity is the explanation for the lifespan shortening at high food concentrations, it is important that daily and lifetime fecundity increase for the increases in food concentrations that decrease lifespan (C). Figure taken from Piper and Partridge (2007).

medium. This method is impractical as it is highly laborious and requires optimisation for DR experimentation.

DR by dilution of a yeast-based medium involves diluting concentrations of dead yeast and sucrose dissolved in an agar medium to which flies have free access (Chapman and Partridge, 1996). In general, as food is diluted from high concentration, lifespan rises to a peak at intermediate nutrient levels through DR, and then falls with further food dilution through starvation (Chapman and Partridge, 1996). There are two possible causes for the reduction in lifespan as the dry food ingredient concentration increases: (A) There is an increase in specific nutrients that reduces lifespan through trade-off effects. Indeed, increased nutrient availability decreased lifespan and increased the daily and lifetime number of eggs laid per female (Chapman and Partridge, 1996). This is consistent with two theoretical models: the first suggests that in times of low nutrient availability, energy is allocated from reproduction to self-preservation in order to survive until resources become favourable again, at which point energy is reallocated to the production of offspring (Shanley and Kirkwood, 2000). The second suggests that the extension of lifespan is the result of a decrease in the cost of reproduction created by decreased fecundity (Barnes and Partridge, 2003). (B) There is an increase in a harmful factor in or of

the food that decreases fly health and lifespan. This factor may be either a specific toxin unintentionally provided in the diet, an increase in the osmotic pressure of the food or an increase in the hardness of the food as a result of increased dry ingredients, which physically prevents the consumption of food.

The reproductive output of the animal can provide an independent indication of the effect of the diet upon health (**Figure 3.1.1**). For (A) to be correct, any decrease in lifespan in response to increased nutrition should be accompanied by increased daily and lifetime fecundity. For (B) to be correct, the increased concentration of a harmful factor should be accompanied by a decrease in both lifespan and fecundity. However, if the food delivers both nutrients to benefit fecundity as well as a harmful effect that reduces lifespan, the phenotype would be indistinguishable from that of DR. It is therefore important to try and distinguish between a toxin-based and a nutrient-based explanation for the lifespan-shortening effect of high food.

Recent work using the agar-medium method suggests lifespan extension in *D. melanogaster* is not a response governed solely by a decrease in calories. Reducing the caloric content of the food fed to flies by lowering the concentration of yeast produced a greater increase in lifespan than the equivalent reduction in calories by lowering the concentration of sugar (Mair *et al*., 2005). This suggests that yeast is the ingredient that has the greatest effect on *Drosophila* lifespan, yet little is understood about the mechanism involved. Furthermore, different laboratories use different sources of yeast and different concentrations of sugar, yeast and agar for DR (Chapman and Partridge, 1996; Kapahi *et al*., 2004; Bross *et al*., 2005). Despite these differences, few laboratories have measured or reported the fecundity of flies at high food concentrations as an indicator of fly health. This lack of fundamental knowledge required for fly DR studies or the lack of convention in fly DR application between labs may have resulted in some of the conflicting reports regarding the response to DR in flies (David *et al*., 1971; Le Bourg and Minois, 1996; Good and Tatar, 2001; Carey *et al*., 2002; Cooper *et al*., 2004). Indeed, some researchers have concluded that fruit flies are an exception to the DR effect (Le Bourg and Minois, 2005).

In this chapter, the lifespan response to increased levels of yeast-based medium was tested to see if it is due to either an energy allocation trade-off effect or a harmful factor. This test was achieved by investigating what effect the osmotic pressure in the food had

on lifespan. In essence, this tests whether the reduction of lifespan reported from increasing food concentration (Chapman and Partridge, 1996) was the result of increased hypertonicity in the fly created by increased dry ingredients in the food. Next, a DR diet that was reproducible between laboratories was established so that the experimental implementation of DR in *D. melanogaster* could be standardised. This involved assembling a range of different yeast-based diets and comparing the lifespan and fecundity of flies on each food type for differences in response to DR. Finally, the method of IF was assessed to see if it could produce lifespan extension in fruit flies.

3.2. Methods

All experiments were carried out on Dahomey wild-type stock grown at standard larval density on standard SY medium (**Chapter 2**). Food was prepared as described in section **2.2.1.** A list of the different ingredients can be found in **Table 3.1**.

Media components		Supplier	Name
100 g[1]	Yeast	Baker's (B.T.P. Drewitt, London, UK)	SYBaker's
		Brewer's (MP Biomedicals, Ohio, USA)	SYBrewer's
		Torula (Borregaard, Sarpsborg, Norway)	SYTorula
		Bacto™ Yeast extract (BD Diag., Maryland, USA)	SYExtract & CSYExtract
50 g	Sucrose	(Tate & Lyle Sugars, London, UK)	
8 g	Cornflour [2]	(B.T.P. Drewitt, London UK)	
10 g	Agar	(Sigma, Dorset, UK)	
3 mL	Propionic acid	(Sigma, Dorset, UK)	
30 mL	Nipagin® M [3]	(Clariant UK Ltd, Pontypridd, UK)	
1000 mL	made to final volume with distilled water		

Table 3.1 Recipe used to make *Drosophila* SY food media.
The values in this table describe the arbitrary reference condition (1.0) used in DR experiments and for rearing flies. Where indicated in the text, the yeast, sugar and agar concentrations were varied.

[1] For yeast comparison experiments, the yeast concentration alone was varied from 10 g/L (0.1) to 200 g/L (2.0)
[2] cornflour (organic polenta) was used for the CSYExtract medium only
[3] solution of 100 g/L methyl 4-hydroxybenzoate in 95% ethanol

3.2.1. Measurements of osmolarity

The osmolarity of SY food medium was determined by a VAPRO 5520 vapour pressure osmometer, and performed to manufacturer's instructions.

3.2.2. Assays with the addition of a water source

Lifespan assays were performed as described in **2.3.5** with the exception that the vials contained either an agar tip or cotton tip. The agar tip was created by filling a 20μL pipette tip with 1% agar solution (containing preservatives as for the SY media) and allowed to set. The stem of the tip was trimmed and the head of the tip was placed into the middle of the SY medium. This allowed flies access to a water source without disrupting the concentration of the SY medium. A pipette tip filled with cotton wool was added to the control treatment. The cotton wool was used to prevent flies from crawling into the pipette tip and becoming trapped.

3.2.3. Fecundity assays

Egg counts were performed as decribed in **2.3.6.** For the sugar concentration experiment, egg counts were performed on days 3, 7, 10, 14 and 21 of treatment. For the first yeast comparison experiment (SYBaker's, SYBrewer's and SYTorula) eggs were counted on days 5, 9, 12, 16, 19, 23, 26, 30 and on days 4, 8, 11, 15, 18, 22, 25, 29 for the second (SYBaker's, SYBrewer's, SYExtract and CSYExtract). Eggs were counted on days 3, 6, 10, 13, 17, 26, 31 and 38 for the water add-back experiment and on days 4, 11, 18, 25, 32, 46 and 60 for the agar concentration range experiment.

3.2.4. Intermittent feeding

Five replicate 1L cages, each containing 100 flies was used for each condition. These cages have two side-arm inlets that can each accommodate a vial. The periods of starvation were initiated at 10:00 (lights-on), whereupon the food vial (2x SYBrewer's) was replaced with an empty vial. In all cages at all times, flies had constant access to a vial containing water that was plugged with wet cotton wool. This was housed in the side-arm not

containing the food vial. In all cases, flies were transferred to fresh food at least three times a week, at which point deaths were scored.

3.3. Results: Dietary restriction by food dilution in *Drosophila*

3.3.1. An example of a harmful factor that produces a response similar to that of dietary restriction

Dietary restriction (DR) can be administered in flies by diluting the concentration of nutrition in its food, while control flies (a.k.a. high fed or fully fed flies) receive food containing ingredients that are greater in concentration. Thus, as described in **Figure 3.1.1**, the differences in lifespan associated with such a regime may mimic that of genuine dietary restriction when in fact the food dilution is merely reducing the amount of a harmful factor that flies are exposed to. Such a flaw can be demonstrated by exposing flies to two different recipes of sugar-yeast (SY) food. One recipe contained the current laboratory yeast (Baker's yeast – 0.05% salt, as w/v NaCl), and the second recipe contained the same yeast from the same supplier but with 0.8% salt added during the manufacturing process 'for taste' (Baker's yeast – 0.8% salt). When flies were exposed to various concentrations of these recipes (**Figure 3.3.1**), fly lifespan in both conditions was observed to increase as food concentration increases from malnutrition, and after reaching a peak lifespan, declines as a result of nutrient-dependent effect of increased nutrition. The Baker's yeast recipe containing 0.05% salt produced a genuine DR response whereby fecundity continued to increase along with increased food concentration. However, the recipe containing the additional salt replicated the scenario in **Figure 3.1.1A**, where fecundity also decreased with increased food concentration. This suggests that additional salt is detrimental to fly health and thus, reducing the food concentration also rescued flies from the toxic effect of the salt.

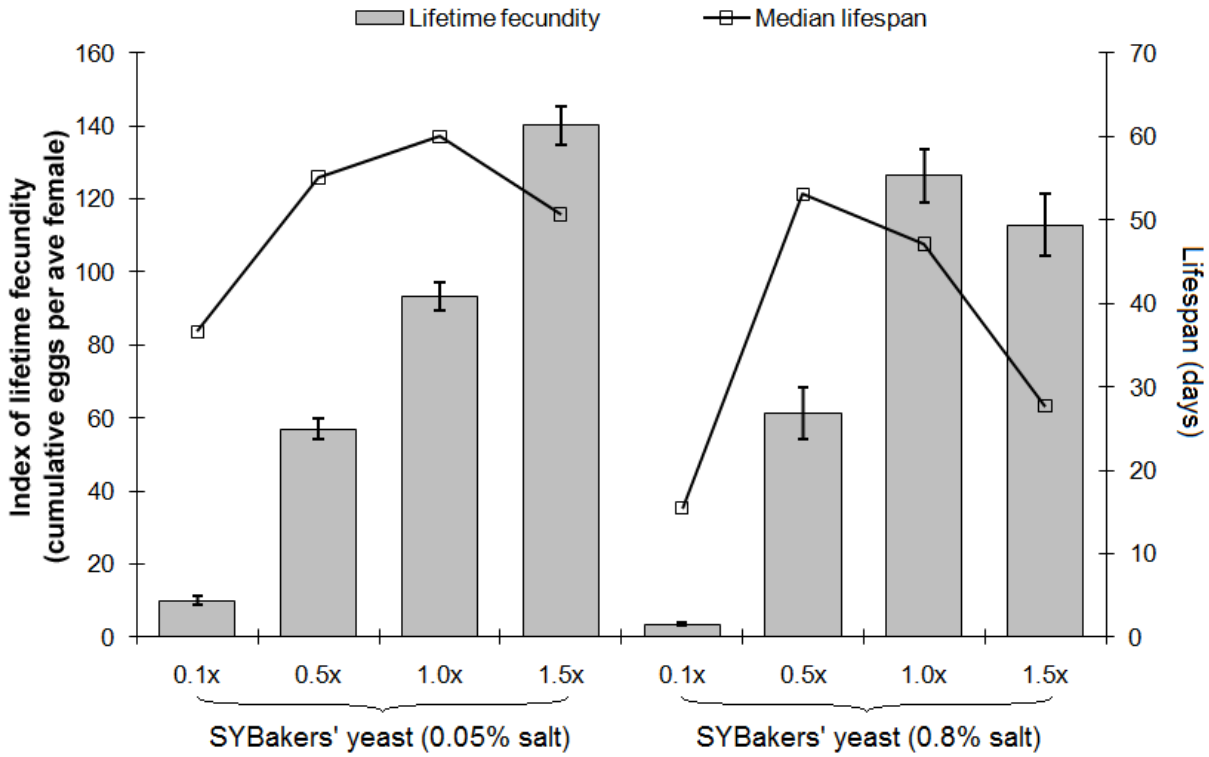

Figure 3.3.1 The responses of lifespan and fecundity to recipes containing 'low' salt and 'high' salt concentrations.
SYBaker's yeast (0.05% salt) produced a genuine DR response due to the increased fecundity coupled with decreased lifespan at an increased food concentration, while SYBaker's yeast (0.8% salt) produced a response reminiscent of an increased toxin at the increased food concentration.

3.3.2. The osmotic pressure of the food medium is proportional to increased sugar and yeast concentration

One candidate mechanism by which increased salt concentration may be detrimental to fly health is that it increases fly dehydration. Thus, one can test whether increasing the nutrient concentration of fly food also increases dehydration in flies. Therefore, the osmotic pressure of three fly SY food recipes containing different dietary yeasts was measured to determine whether the osmolarity of the food was affected by increasing the amount of yeast ingredient in the recipe. Baker's yeast with 0.8% salt, Baker's yeast with 0.05% salt and Brewer's yeast, which is used by some American labs in the field, were all tested. The osmotic pressure of the food medium was found to increase when dry ingredient (SY concentration) increased (**Figure 3.3.2**)[4]. However, the additional 0.8%

[4] Performed in collaboration with Matthew D.W. Piper.

salt in the Baker's yeast increased the osmotic pressure of the corresponding food medium more than the other yeasts.

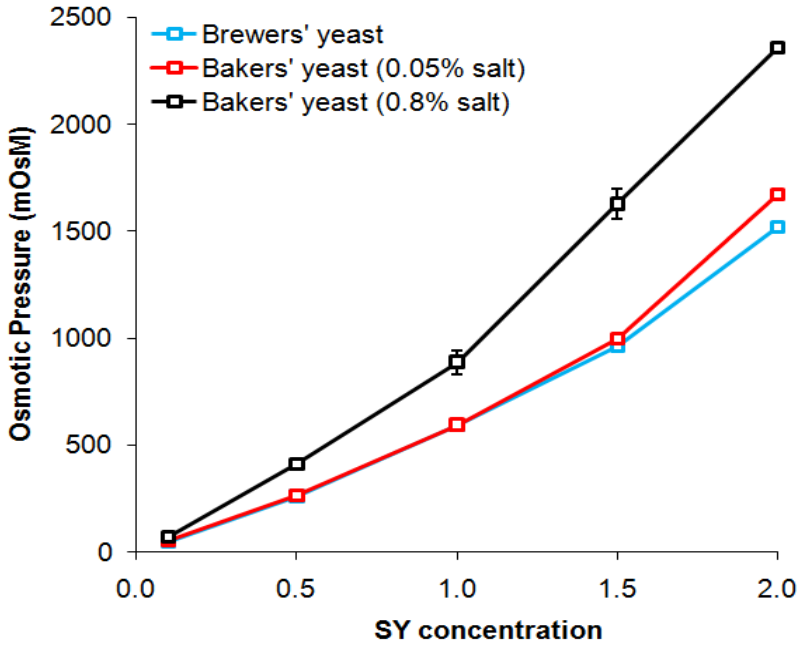

Figure 3.3.2 Three types of dietary yeast were tested for their osmotic pressure.
The Baker's yeast with additional salt (black line) had a greater osmolarity than the other two types of yeast.

3.3.3. Lifespan decreases with increases in osmotic pressure of food medium

The flies were once again tested for their survival on the variety of different SY recipes. However, two additional recipes were compared. One recipe contained Baker's yeast +0.05% salt that had 0.75% salt added during preparation of the food medium to compensate for the salt difference of Baker's yeast +0.8% salt. This tested whether salt was the causal factor behind the increase in osmotic pressure and if it was the cause of the decrease in lifespan. The second recipe contained Brewer's yeast where no additional salt was added.

The overall median lifespan of flies fed diets with additional salt (green and black lines) was significantly lower ($P < 0.0001$, log-rank test) than flies fed diets without additional salt (**Figure 3.3.3**). The median lifespan of flies on all diets increased to an optimum as nutritional availability increased from very low levels, but decreased as the concentration of SY food increased. This was also reflected by a decreasing median lifespan with increasing osmotic pressure of the food. This decreased lifespan was observed at much

greater levels ($P < 0.0001$) with flies maintained on Baker's yeast (0.8% salt) (black line) than flies maintained on the other yeasts (blue and red lines). The Brewer's yeast and the Baker's yeast (0.05% salt) showed no significant difference in lifespan at varying SY concentrations except at 2.0SY where there was significance ($P < 0.005$). The lifespan between any of the yeasts at 0.5SY was not significantly different.

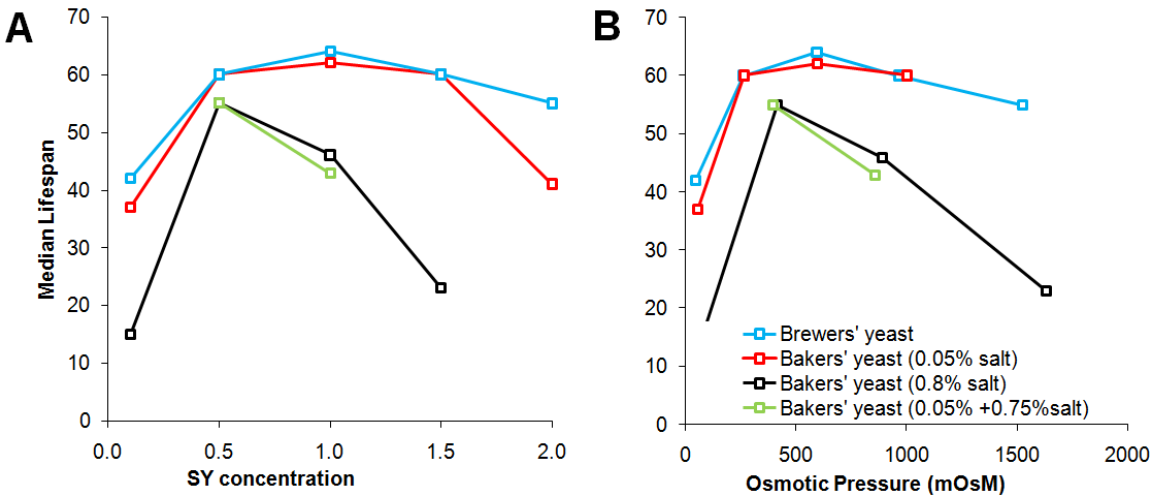

Figure 3.3.3 Median lifespan is dependent on SY concentration and osmotic pressure.
The median lifespan of flies decreases with increasing (A) SY food concentration (B) osmotic pressure. (A) An optimum SY concentration exists for lifespan, which can vary between different yeasts. (B) Different yeasts display different osmotic pressures at which lifespan is optimum.

At a low nutritional level (0.1SY), a greater lifespan was observed in the SY medium containing Baker's yeast (0.05% salt) than in the SY medium containing Baker's yeast (0.8% salt) suggesting the extra salt increases the effect of starvation. The osmotic pressure of Baker's yeast (0.8% salt) at 1.5SY (1600 mOsM) was greater than that of Brewer's yeast at 2.0SY (1500 mOsM). However, the median lifespan of Brewer's yeast 2.0SY flies was still significantly ($P < 0.0001$) greater than flies on Baker's yeast (0.8% salt) 0.5SY. This indicated a specific component in the yeast was detrimental to the flies but did not necessarily cause death by an increased osmotic pressure.

Addition of the difference in salt (0.75% difference) (green line) to the Baker's yeast (0.05% salt) diet (red line) produced a decrease in lifespan similar to the Baker's yeast (0.8% salt) diet (black line). This suggests that salt is the causal factor for the overall lower lifespan in the Baker's yeast (0.8% salt). The increased osmotic pressure of the food and increased detriment to health are likely to be connected. Increased dehydration from

extra salt in the diet is one possible link, thus it would be of great interest to ascertain whether the detrimental effect of additional salt on lifespan could be removed by the addition of a water source in the food medium.

3.3.4. The negative effect of excess salt on lifespan can be rescued by addition of a water source

A water source was provided to flies by the addition of a small volume of agar in a pipette tip to food vials. This enabled flies to actively access water should they require it and self-regulate their hydration. Addition of a water source to experimental vials containing SY medium with Baker's yeast (0.8% salt) showed a rescue in the lifespan of flies on 1.0SY and 1.5SY to the longevity of flies maintained on 0.65SY (**Figure 3.3.4**). This indicated the flies could recover from the water imbalance or osmotic pressure created by increased levels of dry ingredient. This could mean that the lifespan differences observed between food at 0.65SY, 1.0SY and 1.5SY could entirely be accounted for by the water imbalance and there may be no nutrient specific DR effect.

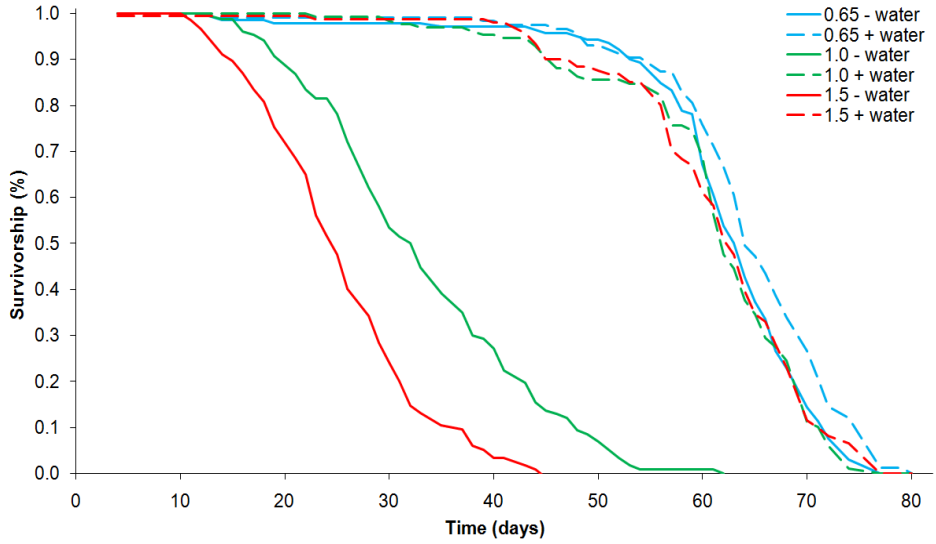

Figure 3.3.4 The survivorship of flies on SY medium containing Baker's yeast (0.8% salt).
Solid lines indicate conditions with no addition of a water source. Dashed lines indicate an addition of a water source.

However, addition of a water source to SY medium made from Baker's yeast (0.05% salt) did not exhibit such dramatic rescues of lifespan (**Figure 3.3.5**). There was no

significant difference in median lifespan between conditions at 1SY (P = 0.748, log-rank test) but there was a marginally significant difference (P = 0.039) in median lifespan between 2SY with added water source and the 2SY control. There was no significance in fecundity between flies with added water source and controls. Flies on 2SY had increased fecundity compared to flies on 1SY. Addition of a water source to SY medium made from Brewer's yeast also did not make a difference to the lifespan or fecundity at 1SY, and also no difference at 2SY food concentration[5]. This suggests that inability to access sufficient free water cannot explain the lifespan shortening effect of high food.

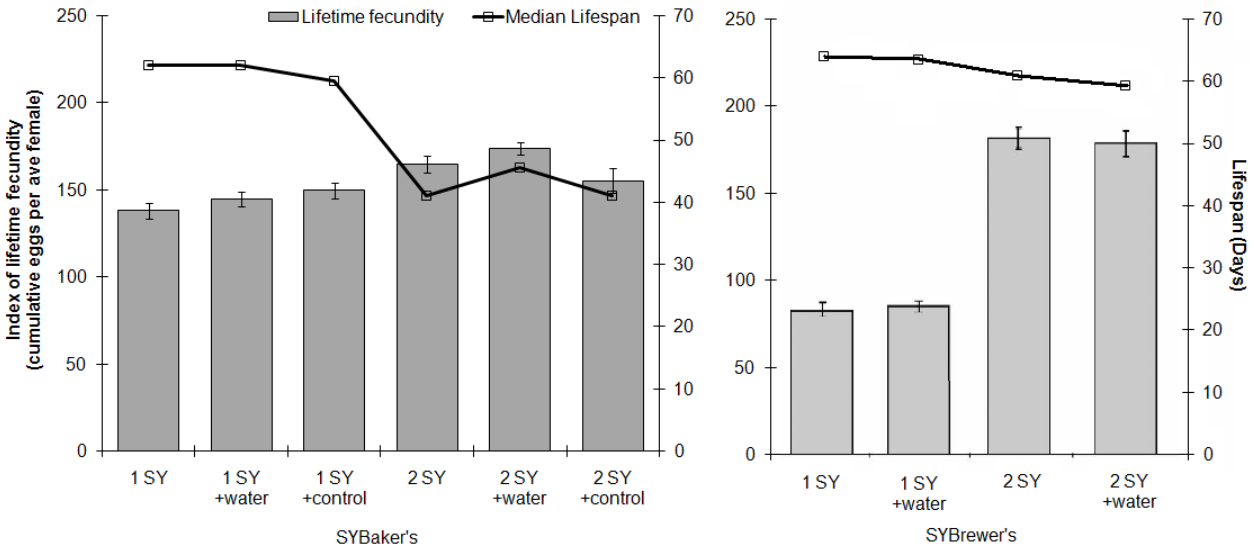

Figure 3.3.5 The effect of adding a water source on the DR response of flies.
Water was added in the form of 1% agar in a pipette tip inserted into the SY food medium containing either Baker's yeast (0.05% salt) (left panel) or Brewer's yeast (right panel). Bars: index of lifetime fecundity ± standard error of the mean; connected black points: median life span.

3.3.5. High levels of dietary sucrose adversely affects fecundity with little effect on lifespan

While it has been shown that the yeast component of a SY diet is critical for DR in *Drosophila*, it is possible that sucrose could also produce life-shortening effects similar to those of yeast if raised to sufficiently high concentrations i.e. higher than those used in

[5] Performed in collaboration with Tim M. Bass, Richard C. Grandison and Matthew D.W. Piper.

Mair *et al*., (2005). To test this, the sucrose concentration in the diet was varied while keeping all other ingredients at a fixed level[6].

Interestingly, there was no requirement for dietary sucrose for maximum fecundity and, surprisingly, addition of sucrose at 100 g/L and higher caused a decrease in female lifespan ($P < 0.00001$, log-rank test) and fecundity ($P < 0.00002$, Wilcoxon rank sum test) indicating that it had a detrimental effect on fly physiology and/ or behaviour. The data shows that sucrose concentrations greater than 50 g/L are not appropriate for DR studies, since elevated fecundity is a required response to nutritional increase. For optimum longevity the flies required the presence of at least 50 g/L dietary sucrose in an SY diet. This is shown in (**Figure 3.3.6**) as a significant increase in median lifespan when sucrose was added to a yeast-only diet (50 g/L versus 0 g/L; $P < 0.00001$, log-rank test). As a result, all experiments reported from here on used a constant 50 g/L of sucrose concentration, as this was neither detrimental to lifespan nor inhibitory to egg-laying.

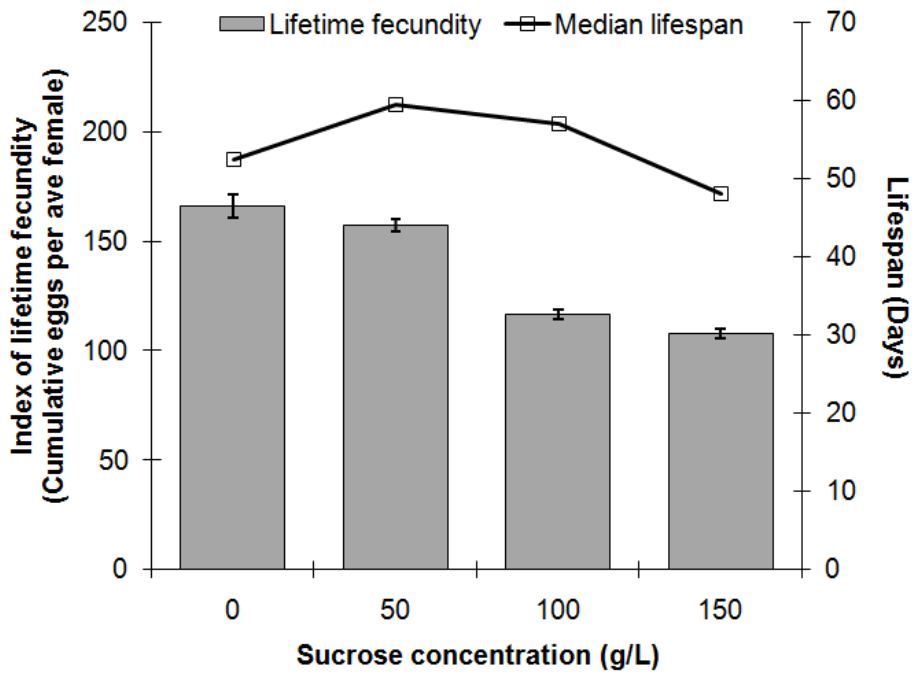

Figure 3.3.6 The effect of dietary sucrose concentration on lifespan and fecundity of mated *Drosophila* females.
Increasing concentrations of sucrose were added to a standard food background of 1.5 SY medium containing Baker's yeast. Over the range of sucrose tested, lifespan was greatest at 50g/L, and a significant decrease in both fecundity and lifespan was observed at concentrations greater than 50 g/L sucrose. Grey bars represent an index of lifetime fecundity (sum of the eggs laid by an average female on the days counted) and connected black points represent median lifespan.

[6] Performed in collaboration with Tim M. Bass, Richard C. Grandison and Matthew D.W. Piper.

3.3.6. Varying the quality of the yeast supply produces a range of effects on lifespan and fecundity

As previously reported in Mair *et al*. (2005), DR lifespan extension can be achieved solely by modulating the yeast component of the diet. The data presented earlier also suggest that the DR lifespan response is not due to a rescue from lack of hydration at high levels of yeast ingredient, because the life shortening effect of high levels of Brewer's yeast could not be rescued by the addition of a water source. Thus, the aim now is to compare the different yeasts used in other DR labs to determine their effects on lifespan and fecundity. Comparisons of fly DR recipes were made from four sources of inactivated yeast: a Baker's yeast, a Brewer's yeast, a torula yeast and an aqueous soluble extract of Baker's yeast[7]. The first three of these are whole-cell lysates, whereas the fourth is a purified extract. These were each used over a range of concentrations from 10g/L (labeled 0.1) to 200g/L (labeled 2.0) while the other media constituents were held constant (**Table 3.1.**).

Comparison of the three whole-yeast food types showed a similar pattern for median lifespan, which gave a peak at 1.0 (100g/L) and lifespan reduction as food concentration was changed above or below this point (three left graphs of **Figure 3.3.7**). Both SYBaker's and SYBrewer's yielded the longest lifespan (69 and 70 days on 1.0 food respectively) while the longest lifespan on SYTorula (63 days at 1.0) was significantly shorter ($P < 0.0001$ in both comparisons, log-rank test). For each of these three yeasts, lifetime fecundity increased with increasing food concentration to 1.5 from which point there was no further increase for SYBaker's and SYTorula. In contrast, fecundity was significantly higher on SYBrewer's when the concentration was raised from 1.5 to 2.0 ($P < 0.001$, Wilcoxon rank sum test). Furthermore, the level of egg-laying on 2.0 SYBrewer's was significantly higher than the peak of any of the other food types tested ($P < 0.005$, 2.0 SYBrewer's versus 1.0 CSYextract and $P < 0.0001$ for all other pairwise comparisons, Wilcoxon Rank sum test). Thus, the limit to higher egg-laying on the other food types was not intrinsically physiological, but was restricted by some feature of the other foods.

[7] Performed in collaboration with Tim M. Bass, Richard C. Grandison and Matthew D.W. Piper.

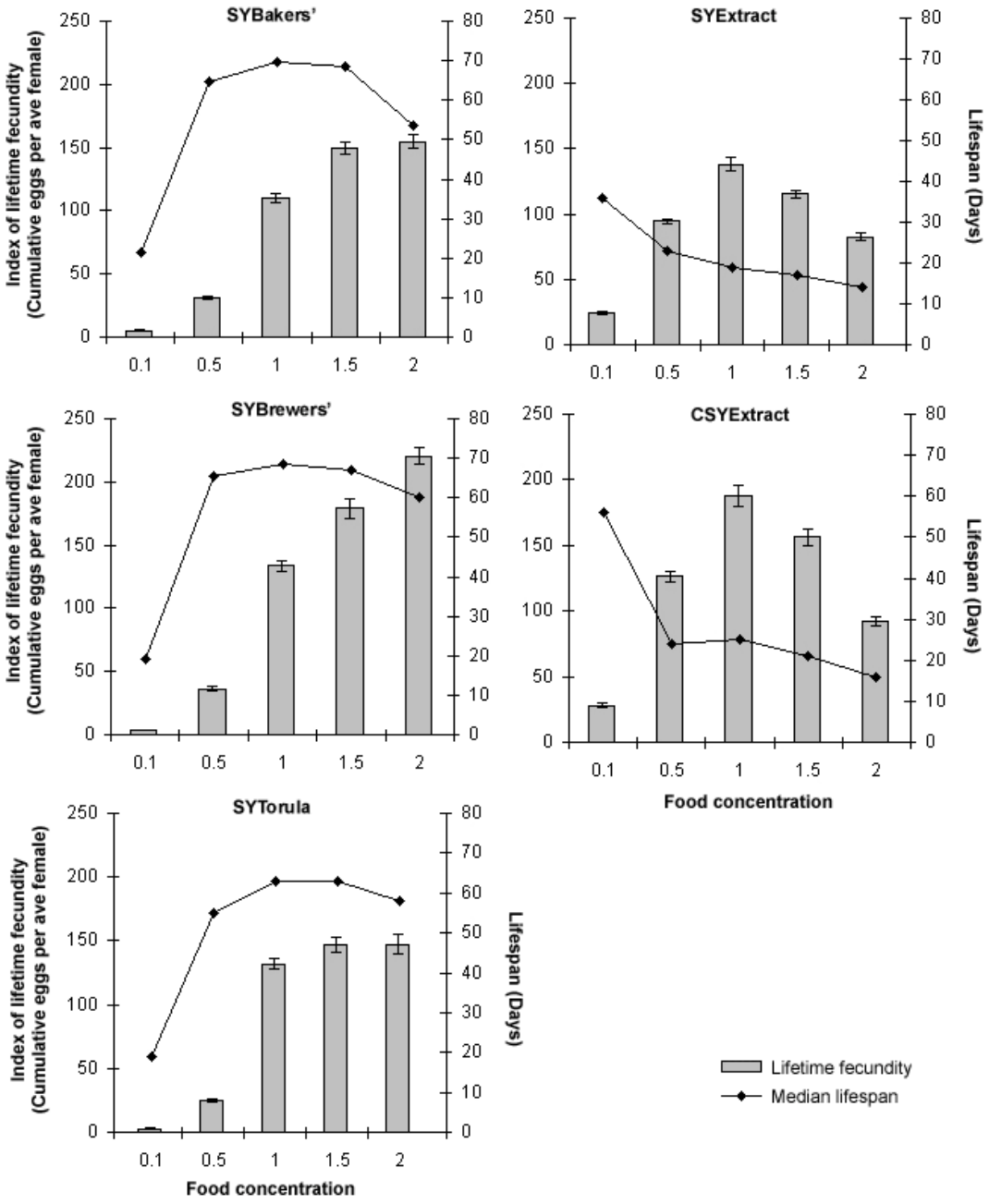

Figure 3.3.7 The effect of a range of concentrations of different commercially available yeasts on lifespan and fecundity.

Five different yeast concentrations were prepared for each of five different sugar-yeast (SY) recipes. SYBaker's, SYBrewer's and SYTorula each refer to food made with different inactivated whole yeast preparations while SYExtract and CSYExtract refer to diets based on an aqueous soluble yeast extract. The nutritional components of each food type were sucrose and yeast or yeast extract and cornmeal (for CSYExtract only). Bars represent an index of lifetime fecundity and connected black points represent median lifespan values.

Yeast-extract-based media produced different lifespan and fecundity responses from the food types made with whole-yeast powders. The most obvious difference was that lifespan decreased for each addition of yeast extract to the medium. This was similar for

CSYExtract and SYExtract (two right graphs of **Figure 3.3.7**), except for 0.1, at which level cornmeal addition resulted in a significantly longer lifespan (36 days on SYExtract versus 56 days on CSYExtract; $P < 0.0001$, log-rank test). Since the positive effect of corn-flour on lifespan was only seen at the lowest concentration of yeast extract (0.1) and the longest lifespan on 0.1 SYExtract was low compared with all other treatments, the data are compatible with an argument that yeast extract resulted in dose-dependent killing. The pattern of lifetime fecundity was similar between SYExtract and CSYExtract, increasing with yeast extract addition to a maximum at 1.0, but decreasing at higher concentrations. Cornmeal addition augmented egg-laying, which peaked at 1.0 CSYExtract at a level similar to that in 1.5 SYBrewer's and higher than the maxima for the other three food types ($P <0.01$ for all pairwise comparisons, Wilcoxon rank sum test). In both the presence and absence of cornmeal, yeast extract was apparently more nutritionally dense than whole-yeast powders since egg-laying was greater on CSYExtract (up to 1.0) and SYExtract (up to 0.5) than the whole yeast diets at corresponding food concentrations. However, fecundity decreased for additions of yeast extract higher than 100 g/L (1.0). Thus, yeast extract at high concentrations is detrimental to fecundity in addition to its negative effect on lifespan.

3.3.7. Increasing the agar concentration in the food medium affects fecundity but not lifespan

Increasing the food concentration could mimic a DR effect purely by increasing the hardness of the food. To test this, the concentration of all food ingredients was fixed at 2.0 SYBrewer's and only the agar concentration was varied (**Figure 3.3.8**)[8]. For each increase in agar concentration, there was a trend towards a decrease in lifetime fecundity. However, this was only significant for the increase from 10 g/L to 15 g/L ($P < 0.0005$, Wilcoxon rank sum test). This reduction was accompanied by a significant increase in lifespan when the agar concentration was raised from 10 g/L to 15 g/L ($P < 0.01$, log-rank test) and a further, non-significant ($P = 0.09$, log-rank test) rise when agar was raised to 20 g/L. These data are consistent with agar controlling food availability in a non-detrimental way between 10 g/L and 20 g/L agar and is therefore explicable as a DR

[8] Performed in collaboration with Tim M. Bass, Richard C. Grandison and Matthew D.W. Piper.

effect. When the agar concentration was further raised to 25 g/L there was no change in median lifespan or lifetime fecundity, but maximum lifespan decreased from 76 days to 71 days. This argues that older flies do indeed differentially suffer if the food becomes sufficiently hard, but for agar concentrations below 20 g/L, food hardness does not on its own cause the life-shortening (DR) effect seen in **Figure 3.3.7**. This indicates that the overall food hardness may adversely affect lifespan.

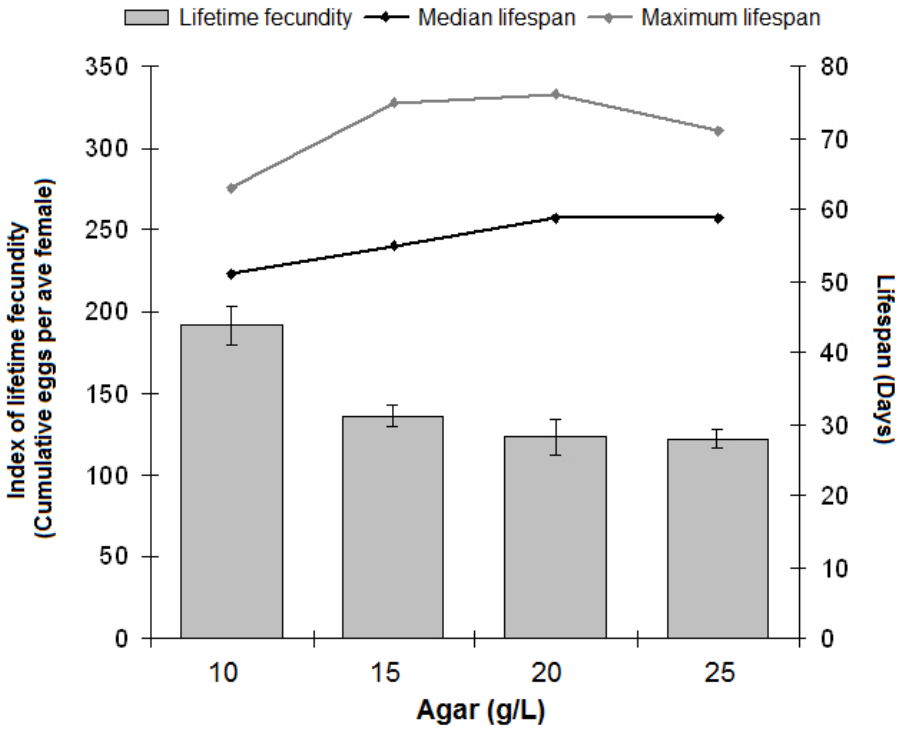

Figure 3.3.8 The effect of varying agar concentration on lifespan and fecundity of females on SYBrewer's medium.
The effect of food hardness on lifespan and fecundity was tested by altering the agar concentration while all other ingredients were held at fixed concentrations (Table 1). This medium contained Brewer's yeast at 200 g/L (2.0 level). Bars represent index of lifetime fecundity, while connected black points represent median lifespan and connected grey points maximum lifespan (median of the last 10% survivorship).

3.4. Results: Dietary restriction by intermittent feeding in *Drosophila*

IF in *Drosophila* has yielded conflicting results as to whether it has beneficial effects on lifespan (Kopec, 1928; Le Bourg and Medioni, 1991). Thus, it would be useful to clarify whether such a method works when flies are fed on the newly optimised yeast diet (SYBrewer's). Dahomey female flies were subjected to daily bouts of either 3h or 6h starvation, during which the flies had access to only water[9]. The treatments had neither a positive or negative effect on lifespan (**Figure 3.4.1.**). While this could be taken to mean that DR does not work in flies, the lack of any effect on lifespan of the more severe restriction makes it impossible to know to what extent the flies were nutrient restricted or whether the periods of starvation were close to adequate to elicit a protective effect. Without a more extensive set of starvation periods, it is not possible to draw definitive conclusions about the effectiveness of this intervention in *Drosophila*.

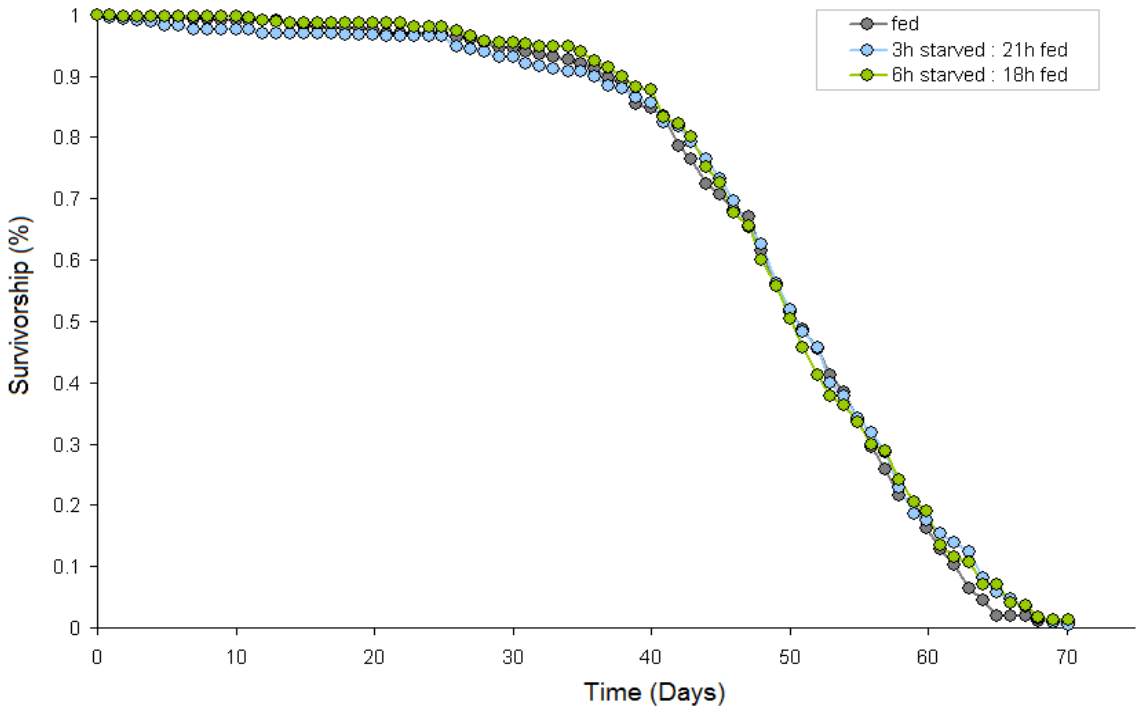

Figure 3.4.1 Intermittent exposure of flies to 2x SYBrewer's food does not increase their lifespan. Throughout adult life, Dahomey females were exposed to daily cycles of starvation: feeding of either 3h: 21h or 6h: 18h. Neither treatment had any effect on lifespan. During the periods of starvation, flies had only access to water.

[9] Performed in collaboration with Tim M. Bass, Richard C. Grandison and Matthew D.W. Piper.

3.5. Discussion

3.5.1. The effect of increased salt in the food medium on fly lifespan

The disposable soma theory of ageing suggests that, when faced with limited resources, an organism's reproductive success is increased by investing only enough resources in maintenance to facilitate reproduction, since somatic tissue is an evolutionary dead-end (Kirkwood, 1977). Dietary restriction extends fly lifespan while reducing fecundity, and two models have arisen to explain why this observation exists. First, life-extension via DR evolved as a mechanism to cope with varying levels of nutrition and represents a shift in the allocation of resources from reproduction to somatic maintenance during food shortage (Masoro and Austad, 1996; Shanley and Kirkwood, 2000). Second, reduced resource availability leads to an increase in lifespan that is mediated by reduced reproduction but that is not a direct result of a trade-off with somatic maintenance (Barnes and Partridge, 2003). In this model, reproduction causes damage that increases mortality. When food supply is restricted there are not enough available resources for reproduction to take place, thus reproductive rate (and the levels of damage it induces) decreases, resulting in lifespan extension. The results described in this chapter suggests that without careful attention to the food composition, studies that claim to be examining extended lifespan due to DR, may simply be studying the rescue of normal lifespan from the effects of toxic food that prematurely shortens life.

Comparisons between Baker's yeast (0.8% salt) and lower salt yeasts (Baker's - 0.05% salt and Brewer's) suggest that excess salt affects lifespan negatively at highly concentrated levels (1.0SY and greater). The osmotic pressure of adult *Drosophila* haemolymph is averaged at 251 mOsM (Singleton and Woodruff, 1994), however *Drosophila* cell lines have been recorded to proliferate successfully in buffers up to 400 mOsM (Wyss and Bachmann, 1976). Therefore, it is not surprising that flies fed SYBaker's yeast (0.8% salt) were observed to be at greater risk from increased osmotic pressure. Further studies are required to distinguish how much of the excess salt in the diet contributes to changes in the osmotic pressure of fly haemolymph, and whether this would be detrimental to the cell *in vivo*.

3.5.2. The effect of increased sugar in the food medium on fly lifespan and fecundity

D. melanogaster in the wild is thought to co-consume fruit material, microbes and yeast from fermenting/ rotting fruit (Spieth, 1974). In the laboratory, flies can be maintained on a combination of sugar, yeast and water (Ashburner, 1989). The data here shows that addition of sugar above 50 g/L to the culture medium was detrimental for both egg-laying and lifespan. This may suggest that fly recipes in DR experiments that manipulated both sugar and yeast were harming fly health at fully fed levels. Thus, lifespan extension observed in DR flies may be a result of rescue from harmful high levels of sugar. These data also indicate that *D. melanogaster* has a low requirement for free sugar for lifespan and fecundity, consistent with the finding that total sugar levels in rotting banana are no more than 20 mM (equivalent to 4.5 g/L sucrose) (Omura and Honda, 2003). Other experiments have shown that *Drosophila* do not modulate their feeding behaviour once sucrose levels rise above 50 mM (Edgecomb *et al*., 1994; Carvalho *et al*., 2005; Mair *et al*., 2005). Thus, the lowered egg-laying observed with high sugar is unlikely to be an effect of reduced feeding, but is likely to reflect an adverse effect on physiology due to the presence of unnaturally high sugar levels, meaning high sugar should be avoided in *Drosophila* DR experiments.

3.5.3. The effect of different yeasts on fly lifespan and fecundity

Increasing additions of one particular Brewer's yeast caused lifetime fecundity to continually rise over a concentration range that also decreased lifespan and so conformed to the expectations of a DR treatment. By changing the yeast from Baker's yeast to Brewer's yeast, a shift occurred in the concentration at which lifespan peaked from 65 g/L yeast (0.65 in Mair *et al*., 2005) to 100 g/L (1.0 shown here). Yeast quality is thus variable. Furthermore, high yeast concentrations that reduce lifespan are not always associated with high fecundity, consistent with the explanation that the lifespan decrease on high food concentrations is not an effect of increased nutrition, but a toxic effect of the yeast. This toxicity could be caused by either: 1) a direct effect of a specific element whose increasing concentration reduces lifespan and perhaps also fecundity, or; 2) an indirect effect of a nutritionally imbalanced diet that results in ill-health. Under the first

explanation, one would expect a pattern of fecundity and lifespan similar to that seen for the flies fed increasing concentrations of yeast extract. In this situation both nutrients and the toxin (e.g. a heavy metal for the sake of discussion) are delivered in the food. This results in increasing fecundity as nutrients increase and toxicity remains below a tolerable threshold (e.g. 1.0 in SYExtract and CSYExtract in **Figure 3.3.7**), beyond which fecundity is reduced. For this same concentration range, lifespan would be ever decreasing. In contrast, toxicity through nutritional imbalance should yield a pattern like that for SYBaker's and SYTorula where the absence of a nutritional component imposes a limit on egg-laying capacity due to depletion from its internal reserves. Previous data on the nutritional requirements of adult *Drosophila* showed that deficiencies for essential amino acids, chloride, phosphorous or calcium reduced egg laying within 16 days, with little effect on the short-term viability of the adult (Sang and King, 1961). Thus a trace element shortfall may limit lifetime egg-laying capacity with little effect on lifespan. An example of this phenotype is shown for flies on 1.5 and 2.0 SYBaker's that have the same level of lifetime fecundity, but markedly different lifespans (**Figure 3.3.7**). Since they both experience the same limitation to lifetime fecundity, the limitation in itself is not what causes shortened lifespan on 2.0. Rather the increasing excess of other dietary components is likely to elevate mortality.

Based on the data presented above, the particular Brewer's yeast used here is the optimal yeast for DR studies with *Drosophila* and now forms the basis for all future laboratory recipes. This also has the advantage of bringing the nutritional content of our yeast in line with that of two other laboratories studying fly DR (Helfand lab and Pletcher lab).

3.5.4. The effect of increased agar in the food medium on fly lifespan and fecundity

The food medium could still mimic a DR effect by producing a harmful effect on lifespan with no adverse effect on lifetime fecundity, which is entirely unrelated to nutrition or a toxin in the food. DR by food dilution results in variable hardness of the food and also variable water availability, and therefore, are the most likely candidates to produce such an effect. The experiments showed that neither could account for the

lifespan-shortening seen when varying the yeast concentration. However, a detrimental effect on maximum lifespan was noted when agar concentration was raised to an extremely high level (25 g/L, more than twice that used for other experiments), which was exacerbated when the yeast concentration was also raised, showing that food hardness can reduce *Drosophila* lifespan. This non-DR based life-shortening effect of hard food is likely to contribute significantly to the studies where yeast and sugar were both raised to 300 g/L and agar to 20 g/L (Bross *et al*., 2005; Zheng *et al*., 2005). The level of agar in future recipes was therefore fixed at 15 g/L.

3.5.5. The optimal recipe for DR implemented by food dilution

The recipe for studies of DR in *Drosophila* for the remainder of the thesis consists of a fixed concentration of 50 g/L sucrose and 15 g/L agar, while yeast concentration can vary from 100 g/L (DR) to 200 g/L (control or fully fed). A consequence of these results is that several published reports concerning fly 'DR' are now no-longer comparable. This may help explain the apparently directly opposing results of Carvalho *et al.,* (2005) and Min and Tatar (2006) on the effect of dietary yeast on feeding behaviour. In light of the comparative data presented here, it is possible decreased feeding behaviour on the yeast extract diet in Carvalho *et al.,* (2005) could be an avoidance response and is not comparable to the increased feeding seen in Min and Tatar (2006) on a diet made from whole yeast lysate. This work highlights the need for dietary uniformity in the fly DR community. The dramatic variability in quality of yeasts from different suppliers also points towards the need for a defined synthetic medium that would avoid the potential problems of toxic effects being introduced from the yeast or its feedstock.

3.5.6. Intermittent feeding did not extend fly lifespan but does not rule out DR in flies

There are several different ways to restrict the access of animals to nutrition and thus extend lifespan by DR (Piper and Bartke, 2008). The results described in this chapter suggest that DR by dilution of yeast in an agar medium is practical and effective. However, food dilution methods are unique to invertebrates, whereas periodic access to

food is used in mammalian studies. One such protocol provides animals with a measured amount of food that is completely consumed before the next meal. While effective for extending rodent lifespan (Weindruch and Walford, 1988), it has been unsuccessful when used on flies (Carey *et al*., 2002; Cooper *et al*., 2004). An alternative technique is EOD feeding, which extends rodent lifespan by alternating periods of access to excess food with periods of starvation. Importantly, the mice subjected to this regime increased their feeding behaviour such that they consumed nearly the same quantity of nutrients as controls (Anson *et al*., 2003). Thus, regular periods without food maybe just as important as reducing nutrient intake for extending rodent lifespan (Goodrick *et al*., 1990). In contrast, this protocol has had little or no success when adapted for flies (Kopec, 1928; Le Bourg and Medioni, 1991). No extension of lifespan using a similar protocol on *Drosophila* (**Figure 3.4.1**) was found, which supports the view that either periods of starvation cannot extend the lifespan of flies (Cooper *et al*., 2004; Le Bourg and Minois, 2005) or the mechanism by which DR extends lifespan is different between flies and mammals. Another possible explanation of these findings is that flies need to maintain a steady food intake if they are to avoid starvation. Periods of food consumption, during which all the food is eaten, interspersed with periods when no food is available may not have the same effect on lifespan as the consumption of the same amount of food at a continuous low rate. Indeed, in the work on intermittent feeding in Mediterranean fruit flies, there were pronounced mortality oscillations linked to food availability, with food removal resulting in acute increases in death rates (Carey *et al*., 1999). While these explanations are possible, the fact that lifespan was not shortened by the severest of restriction treatments means that the determination of the level of nutrient intake reduction, or exactly what other periods of starvation could be protective for lifespan in our flies was not possible. While a more extensive range of starvation periods would be revealing, other factors such as the time of day at which food is removed may also be important since feeding behaviour is controlled by the circadian rhythm (Oishi *et al*., 2004). Thus, it is easy to implement an inappropriate methodology when attempting to DR flies in this way and the absence of a positive result does not rule out the possibility of observing a positive effect using if protocols were optimised.

If nutrient restriction is the critical factor in these DR experiments then intermittent feeding protocols that use different dietary compositions would also be expected to vary

lifespan outcomes in different ways. **Figure 3.5.1** illustrates how this may be possible. When given increasing doses of a relatively concentrated diet (orange line), lifespan would increase as malnutrition lessens to a peak at an intermediate level of food availability. As food availability is increased beyond this point, lifespan decreases via the DR response. At some point, no additional increase in food availability will further shorten lifespan as the organism will reach its limit to ingest more food ('point of satiety' and beyond). If, however, the concentration of the food being provided is low enough (represented by the 'dilute' and 'very dilute' diets in **Figure 3.5.1**), lifespan will increase to a plateau whose onset occurs at the point that the organism's food intake limit is reached. If these dilute food types are used in an intermittent feeding protocol, it would be impossible to find an intermediate level of food exposure which increases lifespan, falsely giving the impression that DR does not exist. It is possible that this can explain why some studies have been published that did not find a DR response (e.g. Harrison and Archer, 1987; Kirk, 2001; Forster *et al*., 2003). As mentioned above, food dilution has proven to be the most successful intervention to implement DR in flies (Piper and Partridge, 2007).

The connection between this intervention, where the food remains in excess, and intermittent feeding can be found by taking the lifespan values at any one level of food availability above the point of satiety in the left panel of **Figure 3.5.1.** A cross-section of these values is shown in the right panel represents the standard DR effect in flies (e.g. **Figure 3.3.7**). It should be noted that in reality, this illustration is somewhat simplistic in that the lifespan-sensitive nutrients represented on the x-axis are unlikely to be accurately represented by the term 'food availability'. Furthermore, nutrient composition variations are likely to alter the point of onset of satiety, which in turn changes the onset of the lifespan plateau. Thus, although further work on diet composition, feeding intervals and measured food availability may uncover an alternative intermittent feeding regime suitable for flies, it is likely to be a labour intensive process that may not provide any more information about DR than dietary dilution.

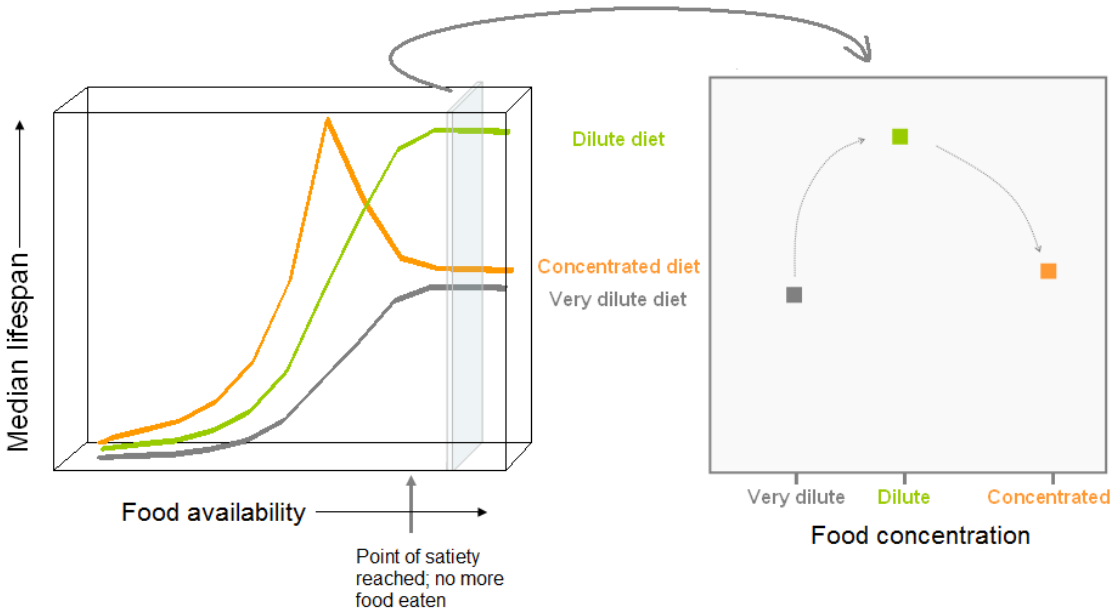

Figure 3.5.1 Model of the relationship between lifespan and DR protocols that reduce access to food either by intermittent exposure (left panel) or nutrient dilution (right panel).
These demonstrate how the composition of food used for intermittent feeding protocols could lead to the false conclusion that DR does not exist for an organism. Three different diets are shown that vary in a given nutrient concentration from 'very dilute' to 'concentrated'. In this example, increasing access to the concentrated diet causes lifespan to rise to a peak (DR) beyond which lifespan decreases. At some point (marked here as the 'point of satiety') the animal will no longer be able to eat any more food, meaning the nutrition level it experiences is capped and no further increase in availability will further decrease lifespan. For the dilute and very dilute diets, the point of satiety is reached before the level of nutrients ingested has a chance to cause lifespan to reduce. Thus, there is no lifespan increase for any intermediate level of food dilution, making it look like the organism does not exhibit a DR response. For flies, these problems can be avoided by assaying lifespan in the presence of excess food that is diluted to differing extents. The relationship of this situation to DR by intermittent feeding is represented by taking a cross-section through the graph on the left. The plot on the right shows the type of data presented herein and for other invertebrate studies. Figure produced by Matthew D.W. Piper.

Chapter 4: Dietary restriction in different genetic strains of *Drosophila*

Abstract

Outcomes of lifespan studies in model organisms are particularly susceptible to variations in technical procedures. This is especially true of dietary restriction (DR), which is implemented in many different ways among laboratories in the fly community. The previous chapter described the effect different yeasts can have on the DR lifespan and fecundity response in the fruit fly Drosophila melanogaster*. In this chapter, the effects of laboratory stock maintenance, genotype differences and microbial infection on the ability of DR to extend lifespan in* D. melanogaster *were studied. None of these factors were found to interfere with the DR effect. These data lend support to the idea that nutrient restriction genuinely extends lifespan in flies, and that any mechanistic discoveries made with this model are of potential relevance to the determinants of lifespan in other organisms. These findings have been published in Grandison* et al. *(2009)(see Appendix 2).*

4.1. Introduction

In order to maximise its reproductive success, an organism must appropriately direct the use of its nutrients to traits such as growth, reproduction and repair. In some circumstances, this may mean selecting one trait at the expense of another depending on nutrient availability (Kirkwood and Holliday, 1979). Consequently, this may result in the phenomenon of dietary restriction (DR), where lowered food intake in an organism can result in longer life but at the cost of reduced rates of reproduction. Despite the effect of DR being widespread (Klass, 1977; Weindruch and Walford, 1988; Chippindale *et al*., 1993; Jiang *et al*., 2000; Partridge and Gems, 2002) and extensively studied since the 1930s (McCay *et al*., 1935), very little is known about the molecular details of exactly what resources are shared in this trade-off and how they are balanced between the traits.

Relatively rapid lifespan experiments using invertebrate model organisms, such as the fruit fly *Drosophila melanogaster*, are likely to help uncover the mechanisms involved. Yet

results derived from these studies are confounded by the effects of non-ageing-related causes of death, such as food toxicity (**Chapter 3**) and differences in experimental protocol between different labs (Kopec, 1928; David *et al*., 1971; Chippindale *et al*., 1993; Chapman and Partridge, 1996; Le Bourg and Minois, 1996; Good and Tatar, 2001; Kapahi *et al*., 2004; Bross *et al*., 2005). For invertebrate studies to be relevant to the study of ageing in higher organisms, it is important to establish techniques that eliminate such effects. Only then can the mechanistic relationship between diet and death be established, providing modes of action to be tested in the longer lived models.

The experiments described in chapter 3 showed that different dietary yeasts in the food medium can have a major effect on the fly response to DR, and by following a systematic approach, the dietary composition was optimised such that fecundity and lifespan are maximised and any non-specific adverse effects of the food are avoided. However, such optimisation is only applicable to studies that involve the *Drosophila* strain, Dahomey. Different fly strains are used for studies of DR in other laboratories in the field, and thus, the DR response to the particular food recipe described in chapter 3 is unknown in these differing genetic backgrounds. Furthermore, different techniques are used to maintain the fly strains, which can result in inbreeding or selection for early reproduction, which is known to shorten lifespan (Luckinbill *et al*., 1984; Rose, 1984; Sgro and Partridge, 1999).

Another possible factor that may cause variation in DR studies is the presence of *Wolbachia*, a genus of gram-negative bacteria that form intracellular infections in many arthropod species (Werren, 1997), and are found in a large number of *Drosophila* laboratory stocks (Clark *et al*., 2005). The bacterium is transmitted by females only via the egg cytoplasm and is known to induce male killing, parthenogenesis, cytoplasmic incompatibility and feminization, processes that alter host reproduction and consequently increase its representation within a population (Charlat *et al*., 2003). Furthermore, one particular strain of *Wolbachia* has been shown to be highly detrimental to fly health (**Figure 4.1.1**) (Min and Benzer, 1997), and more recently, *Wolbachia* has been shown to have an effect on the longevity of *Indy* flies, a mutant previously reported to be long-lived (Toivonen *et al*., 2007). Thus, it would be an advantage to assess the *Wolbachia* status in the different lines used in DR studies and the role, if any, *Wolbachia* has on DR lifespan.

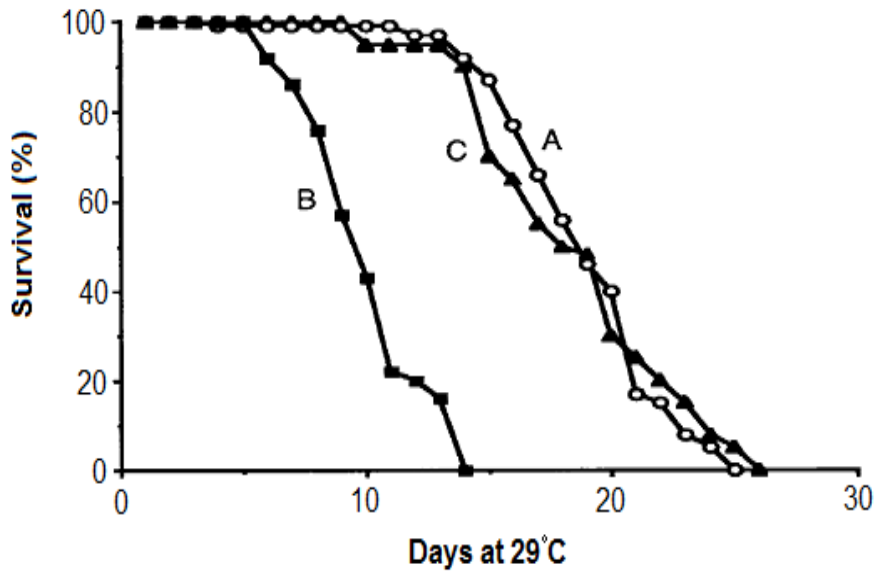

Figure 4.1.1 Reduction of lifespan by *Wolbachia* infection, and recovery by tetracycline-treatment.
(A) Survival curve of uninfected control flies (*white*^{1118}). (B) *white*^{1118} flies after infection with *Wolbachia*. (C) Cured by tetracycline treatment. Each initial population was 100. These curves were obtained by culture at 29°C. The effects at 25°C are similar, with all lifetimes longer by a factor of 2. Figure taken from Min and Benzer (1997).

In this chapter, the effect of different techniques of long-term stock maintenance and microbial infection on the responses of 'wild-type' laboratory-maintained flies to DR was examined. These experiments were undertaken in order to establish a working protocol that avoids laboratory artefacts and will therefore aid studies seeking the molecular mechanisms of DR. As a result of performing these experiments with flies of different genetic backgrounds, interesting differences were found in the interaction between diet and genotype that form a solid basis for future work to uncover how DR extends the lifespan of flies and other organisms.

4.2. Methods

4.2.1. Fly stocks

w^{Dahomey} was generated by backcrossing the *white* (*w*) gene from *w*^{1118} into the Dahomey genetic background. It has since been maintained in one large population cage with a feeding regime as described in section 2.2.1 for Dahomey. *w*^{1118}, *yw*, OregonR and Canton Special (CantonS) have been maintained in the lab for many years under a variety of conditions. Generally, this involves transferring each new generation to a fresh set of

several half-pint bottles or vials of food. These are usually kept at 18°C to extend each generation's lifecycle and are fed 1x SY food (**4.2.2**).

4.2.2. Food media

Food was prepared as described in section 2.2.1. Flies were fed on SYBrewers diet (**Chapter 3**), where 1 litre of SYBrewer's (1.0) contained: 100g autolysed Brewer's yeast (MP Biomedicals, Solon, OH), 50g sucrose (Tate & Lyle sugars, London, UK), 15g agar (Sigma, Dorset, UK), 3mL propionic acid (Sigma, Dorset, UK), 30mL Nipagin M solution (100g/l methyl 4-hydroxybenzoate in 95% ethanol) (Clariant UK Ltd, Pontypridd, UK), and distilled water up to 1L.

In dietary restriction experiments, flies were fed differing concentrations of yeast while all other ingredients were kept constant. SYBrewer's (0.1) contained per litre: 10g Brewer's yeast, 50g sucrose, 15g agar, 3mL propionic acid, 30mL Nipagin M solution, and distilled water up to 1L. SYBrewer's (0.5) contained per litre: 50g Brewer's yeast, 50g sucrose, 15g agar, 3mL propionic acid, 30mL Nipagin M solution, and distilled water up to 1L. SYBrewer's (1.0) contained per litre: 100g Brewer's yeast, 50g sucrose, 15g agar, 3mL propionic acid, 30mL Nipagin M solution, and distilled water up to 1L. SYBrewer's (1.5) contained per litre: 150g Brewer's yeast, 50g sucrose, 15g agar, 3mL propionic acid, 30mL Nipagin M solution, and distilled water up to 1L. SYBrewer's (2.0) contained per litre: 200g Brewer's yeast, 50g sucrose, 15g agar, 3mL propionic acid, 30mL Nipagin M solution, and distilled water up to 1L.

4.2.3. Lifespan and fecundity assays

Lifespan assays were performed as described in section 2.3.5. For fecundity measurements, eggs were counted after the flies had been in the vials for between 18 and 24h. Generally, these counts were performed once a week for the first six to seven weeks of adult life. Importantly, the first egg count was only conducted after at least four days exposure to the new food in order to allow time to adjust to the new nutritional conditions.

4.2.4. Tetracycline treatment

Tetracycline treatment was performed by the addition of 25 μg/ml tetracycline during the cooking process to 1x SY food, and maintaining flies on the food for two generations. After treatment, lines were returned to tetracycline-free food for at least five generations before experiments were performed.

4.2.5. PCR detection of *Wolbachia* infection

Genomic DNA extraction, PCR and gel electrophoresis was performed as described in section 2.5. PCR for detection of *Wolbachia* was performed using primers wsp81F (5' TGG TCC AAT AAG TGA TGA AGA AAC) and wsp691R (5' AAA AAT TAA ACG CTA CTC CA) (a kind gift from G. D. D. Hurst) as described in Zhou *et al.*, (1998). These primers were shown to be able to amplify the *wsp* gene fragment in a number of different *Wolbachia* strains tested in Zhou *et al*., (1998). These primers amplify a DNA fragment ranging from 590 to 632 bp depending on the individual *Wolbachia* strain. In each case, a sample of flies form the experimental generation was used for PCR testing.

4.3. Results

4.3.1. Comparison between different laboratory strains on standard 1x SYBrewer's media

The outbred laboratory strain of *Drosophila*, Dahomey, was used for all of the DR optimisation experiments described in chapter 3. This strain has been maintained for many years on an SY diet in large population cages with overlapping generations. In contrast, most laboratory wild-type strains are largely inbred and maintained in relatively small numbers in individual containers and may have a varied nutritional history. Some of these housing conditions can easily lead to selection for early reproduction, which is known to cause shortened lifespan (Luckinbill *et al*., 1984; Rose, 1984; Sgro and Partridge, 1999). The lifespan of several commonly used wild-type *Drosophila* strains was assayed

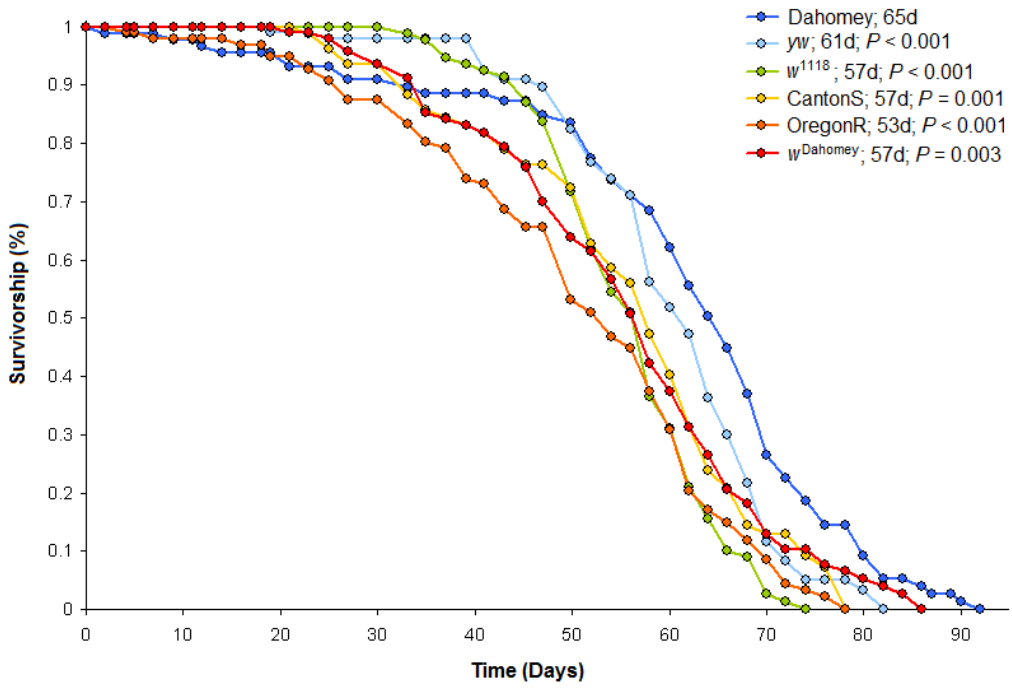

Figure 4.3.1 Different laboratory strains of wild-type *Drosophila* have different lifespans.
Each genotype was raised in parallel under the same conditions and assayed on 1x SY for lifespan. All strains that were tested exhibited a shorter lifespan than our outbred laboratory strain Dahomey. The graph legend reports the strain name; median lifespan in days and; *P*-value from the log-rank test when compared to Dahomey.

on the newly optimised 1x SY food (**Figure 4.3.1**)[10]. In all cases, the lifespans were significantly shorter than that of Dahomey and exhibited median lifespans from 53 days for OregonR to 65 days for Dahomey.

4.3.2. Comparison of the DR response between different laboratory strains

The different strains were next subjected to varying SY food concentrations to both assess how the new DR protocol is likely to behave when implemented in other laboratories that routinely use fly stocks other than Dahomey, as well as to look for strains with altered DR responses that might provide insights into its mode of action[11]. The operational definition of DR is the range of nutrition that causes lifespan to increase and fecundity to decrease (Partridge *et al*., 2005b). It should be noted that this definition excludes the dilution from 0.5x down to 0.1x, as this caused the flies to become malnourished and both lifespan and fecundity to decrease (**Figure 4.3.2**). For Dahomey

[10] Performed in collaboration with Richard C. Grandison, Tim. M. Bass and Matthew D.W. Piper.
[11] Performed in collaboration with Richard C. Grandison, Tim. M. Bass and Matthew D.W. Piper.

and $w^{Dahomey}$, the DR range was from 2x to 0.5x food, while for w^{1118} and CantonS it was from 2x to 1x, and for OregonR was from 1.5x to 0.5x. For OregonR only, the highest food concentration caused egg laying to decrease, which indicated that the associated lifespan decrease from 1.5x to 2x was not accompanied by increased intake of biologically valuable nutrition and therefore could be due to a non-specific detrimental effect of high food. It was thus considered outside of the functional DR range for this strain. Finally, for *yw*, there was a clear DR response from 1x to 0.5x food but owing to incomplete data, any possible broader DR effect cannot be reported. Thus in all cases, a DR response was observed under these conditions although its exact nature was different for different wild-type strains.

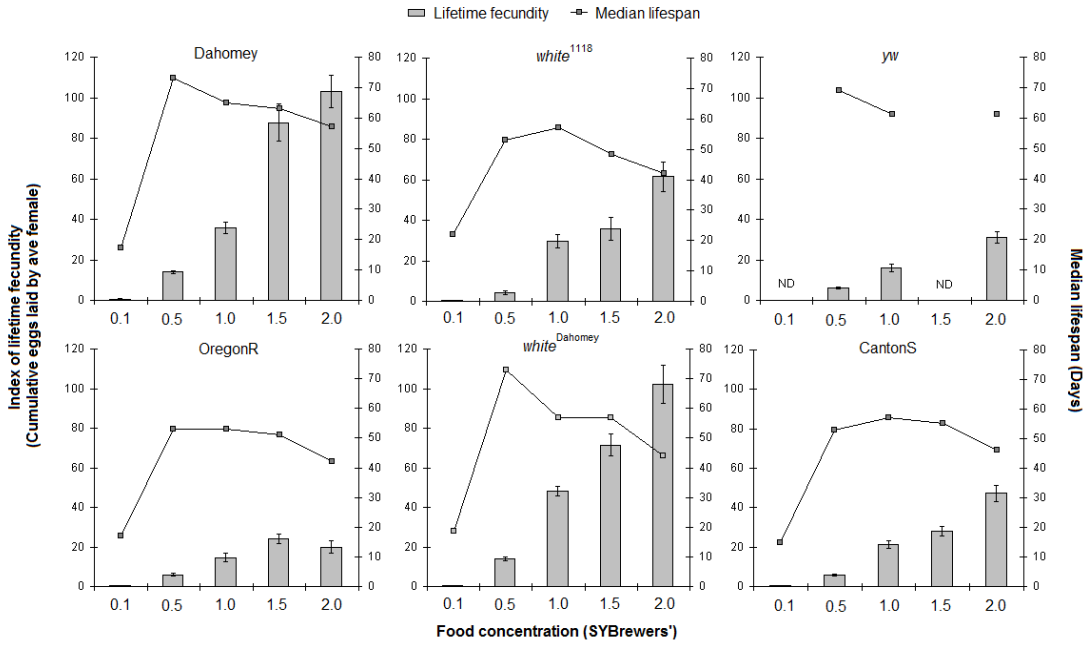

Figure 4.3.2 Different laboratory strains subjected to DR.
When tested in parallel under the same conditions, all wild-type strains tested exhibited a DR response. This is defined as a simultaneous increase in lifespan and decrease in lifetime fecundity when nutrient availability was reduced. Bars: index of lifetime fecundity ± standard error of the mean; connected points: median lifespan in days; ND: not determined. Data shown are from a single trial in which all lifespans were run simultaneously. They are representative of triplicate data sets for Dahomey, CantonS and OregonR and duplicates for yw; w^{1118} and $w^{Dahomey}$ data are from a single trial.

In all comparisons from all trials, Dahomey, $w^{Dahomey}$ and *yw* exhibited the longest lifespan (**Table 4.1**) with medians from 69 to 73 days over different trials on 0.5x food (Dahomey vs $w^{Dahomey}$, $P = 0.69$; Dahomey or $w^{Dahomey}$ vs highest median lifespan from each other genotype, $P < 0.001$, log-rank test). Dahomey and $w^{Dahomey}$ also exhibited

higher reproductive output than the other wild-types at each food concentrations except 0.1x, as well as the maximum reproductive output from all conditions (on 2x food) (Dahomey vs $w^{Dahomey}$, $P = 0.97$; Dahomey or $w^{Dahomey}$ vs highest reproductive output for each other genotype, $P < 0.003$, Wilcoxon rank-sum test).

4.3.3. Effect of tetracycline treatment on the DR effect

Drosophila are host to a range of microbes including a bacterium of the genus *Wolbachia* that resides in the cytoplasm of reproductive tissues of many different fly strains (Werren, 1997). In some cases, the presence of *Wolbachia* has been shown to alter lifespan (Min and Benzer, 1997). Recently, a vertically inherited factor that was curable by tetracycline treatment was shown to account for at least part of the long lifespan of a long-lived *Drosophila* mutant (Toivonen *et al*., 2007). Thus, it was important to examine the effect of such infections on DR, because if they account for the lifespan difference, it is unlikely DR in *Drosophila* is useful as a model for higher organisms.

Different wild-type strains were tested by PCR detection of the gene for *Wolbachia* surface protein (wsp). All except w^{1118} and OregonR were infected (**Figure 4.3.3A**). Thus, *Wolbachia* infection *per se* cannot account for the full effect of nutrition on lifespan because all the strains exhibited a DR response.

To test if tetracycline-treatment could eliminate the DR response by other means, we selected three lines for treatment (Dahomey, CantonS and OregonR)[12]. After two generations on food that contained tetracycline, flies were subsequently maintained on normal food to recover for at least five generations. PCR testing revealed that the treatment was effective as both Dahomey and CantonS were cleared of *Wolbachia* (**Figure 4.3.3B**). When subjected to different food concentrations, all three tetracycline-treated lines retained their DR response (**Figure 4.3.3C**). In the trial shown, the lifespan peak for all three strains was at 1x food and fecundity increased to 2x food. While this was qualitatively different from that seen in the previous trials with non-tetracycline-treated flies, a further trial with these lines after an additional five generations on normal food, revealed more similar data to that shown in **Figure 4.3.2** (data not shown). Thus,

[12] Performed in collaboration with Richard C. Grandison, Tim. M. Bass and Matthew D.W. Piper.

tetracycline-treatment may produce a transitory alteration in the way flies respond to food, but its effects cannot account for the DR response.

Wild-type strain	Food conc (x)	Median lifespans[13]			Ave lifespan change due to DR[14]
		Trial 1	Trial 2	Trial 3	
Dahomey	0.1	17.1	18	ND	
	0.5	73	73.5	69.1	
	1	65	66.5	59.5	31%
	1.5	63	64	48	
	2	57	55	52.5	
yw	0.1	ND	ND	ND	
	0.5	69	ND	73.5	
	1	61	ND	66.5	12%
	1.5	ND	ND	ND	
	2	61	ND	48	
w^{1118}	0.1	22	ND	ND	
	0.5	53.1	ND	ND	
	1	57	ND	ND	36%
	1.5	48.4	ND	ND	
	2	42	ND	ND	
CantonS	0.1	15	18	ND	
	0.5	53.1	57[15]	50	
	1	57	59.5	48	28%
	1.5	55.1	52.5	38.5	
	2	46.1	45.5	38.5	
OregonR	0.1	17	22	ND	
	0.5	53.1	45.5	66.5	
	1	53.1	52.5	59.5	15%
	1.5	50.9	48	55	
	2	42	45.5	52.5	
$w^{Dahomey}$	0.1	18.5	ND	ND	
	0.5	73	ND	ND	
	1	57	ND	ND	66%
	1.5	57	ND	ND	
	2	44.1	ND	ND	

Table 4.1 Summary of median lifespan in different wild-type strains on different foods.

[13] red numbers denote the greatest median lifespans and blue numbers the shortest median lifespans, within the DR food range for that strain in that trial.

[14] For all DR ranges for each strain, the longest-lived condition was significantly different from the shortest-lived condition; percentages are derived from the average lifespan difference due to DR.

[15] In cases where there was no significant difference between two food types for the longest or shortest-lived condition, two numbers are coloured red or blue.

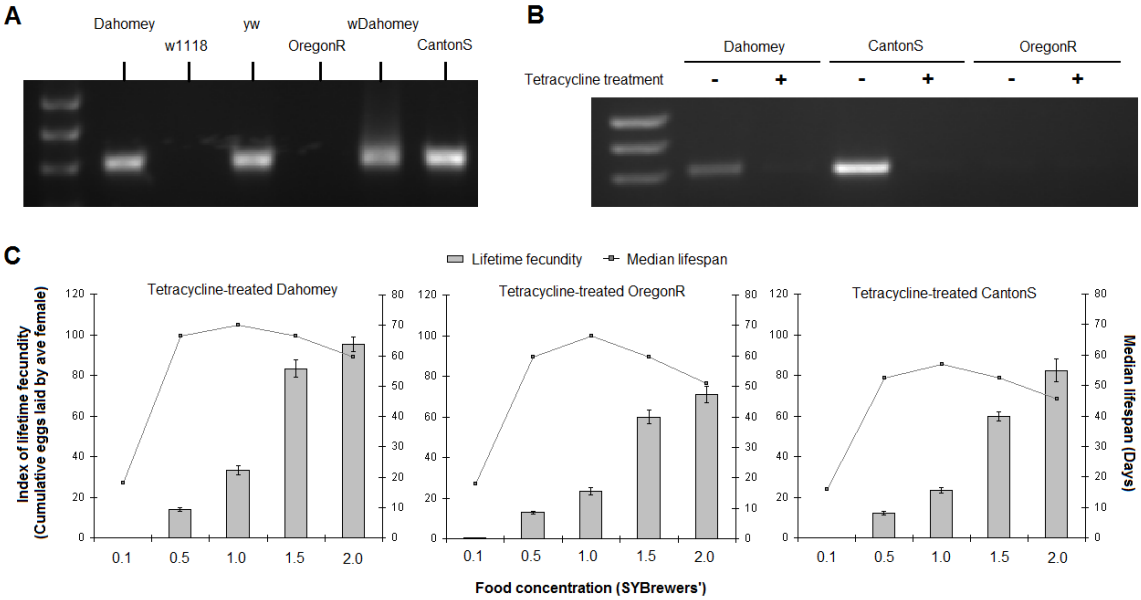

Figure 4.3.3 Tetracycline treatment does not eliminate the DR response.
(A) Gel showing diagnostic PCR for the presence of the intracellular bacterium *Wolbachia*; (B) three strains were selected from the set of wild-types for treatment with tetracycline, which was sufficient to clear *Wolbachia* if present. (C) Each of the three strains was then allowed at least five generations to recover on non-tetracycline-containing food before being assayed for lifespan and fecundity on different concentrations of food. Each of the three strains still exhibited a DR response after tetracycline treatment. Bars: index of lifetime fecundity ± standard error of the mean; connected points: median lifespan in days. Data shown are from one of two trials in which all lifespans were run simultaneously.

4.4. Discussion

4.4.1. Dietary restriction in *Drosophila* does not appear to be a laboratory artefact

For ease of handling and to extend generation times, fly stocks in the laboratory are often kept in small numbers, under relatively poor nutrient conditions and at low temperatures. Over time, these factors are likely to exert selective pressures that could influence lifespan. Importantly, when transferring stocks to fresh food for maintenance, it is relatively easy to select for early age of reproduction, which is known to reduce adult lifespan (Fowler and Partridge, 1992; Sgro and Partridge, 2000). That this happens in the laboratory has been demonstrated by comparing the lifespans of flies maintained for years in the laboratory under normal stock-handling conditions with others selected for early or late reproduction as well as others freshly caught from the wild (Promislow and Tatar, 1998; Linnen *et al*., 2000; Matos *et al*., 2000; Sgro and Partridge, 2000). This study

showed that the laboratory stocks were as short-lived as those selected for early reproduction, while the wild-caught lines had a much longer lifespan, similar to flies selected for late reproduction. A wild-type outbred stock (Dahomey) has been maintained in the laboratory since 1970 in large population cages with overlapping generations. When compared with other laboratory wild-type strains maintained in the Partridge laboratory using routine stock handling techniques, Dahomey demonstrated the capacity for both the longest lifespan and the greatest lifetime egg-laying output (**Figure 4.4.1**). This suggests that the maintenance of flies using large population cages with overlapping generations appears to preserve the life history characteristics of wild-flies for long periods of time. This is in agreement with previous work that demonstrated this fact for flies maintained in the laboratory during a three year period (Sgro and Partridge, 2000). Another explanation may be that the food has been optimised for maximal lifespan and fecundity in Dahomey flies, and thus, may not be optimal for flies of different genetic backgrounds.

Importantly, despite the differences between strains in their selection histories, all exhibited a DR response (**Figure 4.3.2**). This is in agreement with a recent study of several wild-derived strains of *C. elegans* showed that all exhibited a DR response (Sutphin and Kaeberlein, 2008). However, it has also been proposed from work with mice that lifespan extension by DR could simply be an artefact of laboratory domestication, because a wild-caught strain was reported whose longevity was not increased in response to a typical DR regime (Harper *et al*., 2006). Thus, one important experiment would be to directly test flies recently caught from the wild for the lifespan extending effects of DR. It should be noted that the invertebrate studies were conducted using a DR technique that deprived worms of bacteria, while the rodent study used a food restriction protocol with only one level of limitation. Thus, as described in chapter 3, diet design and an incomplete range of food concentrations could be important factors in explaining why the DR effect was apparently absent from wild mice (Harper *et al*., 2006).

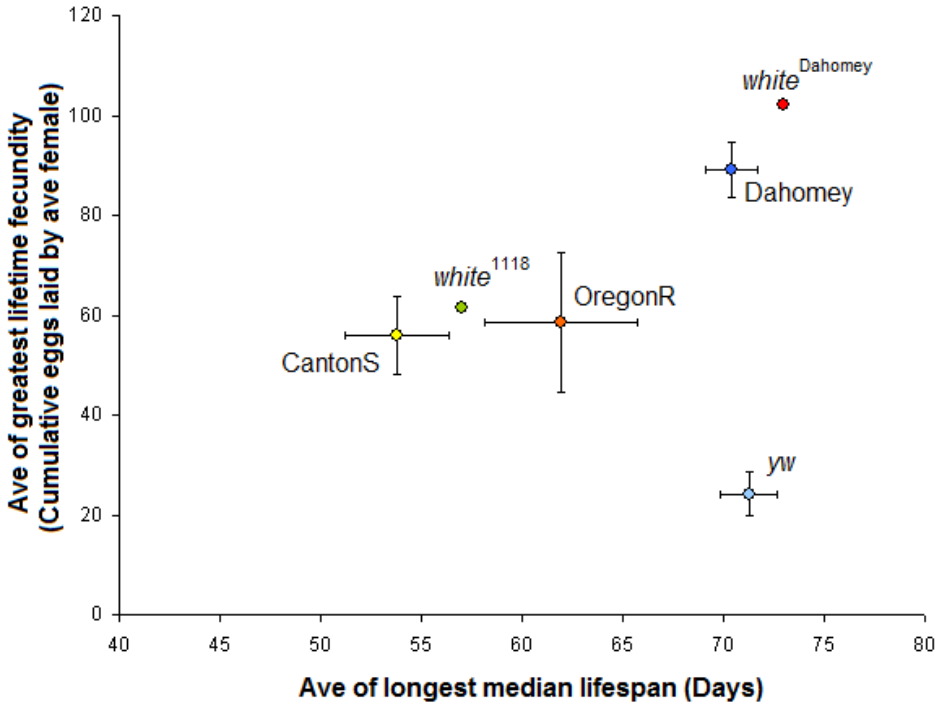

Figure 4.4.1 The Dahomey genetic background is capable of the longest lifespan and greatest reproductive output of the wild-type strains tested.
For median lifespan, the data are the averages from the longest lived conditions for each strain. For lifetime fecundity they are the average of the condition producing the greatest lifetime reproduction. It should be noted that the conditions under which these occur is different for the two traits, as predicted by the expectations of DR, and that they may be different for each different strain. Data from *N* independent repeats, where *N* = 5 for Dahomey, CantonS and OregonR; *N* = 2 for yw, and; *N* = 1 for w^{1118} and $w^{Dahomey}$.

4.4.2. Dietary restriction in *Drosophila* is not sensitive to tetracycline treatment, but varies with diet quality and genotype

This chapter shows that DR is not sensitive to infection with the bacterium *Wolbachia*, or indeed any other tetracycline-sensitive infection that may be present in flies (**Figure 4.3.3**). Interestingly, the levels of fecundity at a given food concentration differed after tetracycline treatment (compare **Figure 4.3.2** with **Figure 4.3.3C**). This indicates that some tetracycline-sensitive microbes carried by some flies might be involved in the control of fecundity. Indeed, some evidence has been provided to suggest that *Wolbachia* reduces the fertility of infected male *Drosophila*, which may consequently affect female fecundity (Snook *et al*., 2000).

The data presented here extend the work performed in chapter 3 to optimise a DR protocol to avoid lifespan variations from non-nutrient dependent effects. In this chapter,

the food concentration to yield the longest lifespan in Dahomey before tetracycline treatment was at 0.5x (**Figure 4.3.2**), whereas after tetracycline treatment the longest lifespan food concentration was found to be at 1x. In chapter 3, the peak lifespan was reported at 1x. This suggests that it is unlikely that the shifts in food concentration for the peak lifespan originated from tetracycline treatment. Rather it may demonstrate an inherent problem with using a natural ingredient like yeast whose nutritional content may vary seasonally due to production methodology and the quality of its feedstock. In doing so, it also highlights the need for a standardised synthetic defined medium to replace yeast-based diets to study the details of how lifespan varies with food composition. Interestingly, not all strains exhibited a lifespan peak at the same food concentration as Dahomey (**Figure 4.3.2**). It is already known that genotype can affect the interaction between lifespan and food (Clancy *et al*., 2002; Giannakou *et al*., 2008; Min *et al*., 2008; Piper and Bartke, 2008) and could indicate the breadth of the DR effect on fly health. One interesting possibility from these data is that if flies of different genotypes die from different pathologies, DR has the ability to delay the onset of each of these causes of death, which agrees with data from rodent studies (Maeda *et al*., 1985; Weindruch and Walford, 1988). Future work on the exact molecular mechanisms of DR via interactions with different genotypes on precise dietary manipulations will be the key to exploring this further.

Chapter 5: Measuring food intake in *Drosophila melanogaster*

Abstract

Measurement of food intake in the fruit fly Drosophila melanogaster *is often necessary for studies of behaviour, nutrition and drug administration. There is no reliable and agreed method for measuring food intake of flies in undisturbed, steady state, and normal culture conditions. Current methods, based on food labels, cannot be used to measure undisturbed feeding rate because they fail to take into account changes in the capacity of the fly for the label. An alternative method based on measurement of feeding behaviour by proboscis-extension, when validated by short-term measurements of food dye intake, provides an accurate indicator of food consumption under undisturbed experimental conditions. The method accurately demonstrated that (a) female flies feed more frequently than males, (b) flies feed more often when housed in larger groups and (c) fly feeding varies at different times of the day. It also showed that alterations in food intake are not induced by dietary restriction or by a null mutation of the fly insulin receptor substrate* chico*. In contrast, mutation of* takeout *increases food intake by increasing feeding frequency while mutation of* ovo^D *increases food intake by increasing the volume of food consumed per proboscis-extension. This approach provides a practical and reliable method for quantification of food intake in* Drosophila *under normal, undisturbed culture conditions. These findings have been published in Wong* et al. *(2008) and Wong* et al. *(2009)(see Appendices 3 and 4).*

5.1. Introduction

As described in chapter 3, DR is achieved in *Drosophila melanogaster* by reducing the quality (nutrient concentration) of the food given to the flies with the quantity maintained in excess of that which they can consume (Chapman and Partridge, 1996). Despite the fact that fecundity correlates with food medium concentration (Chapman and Partridge, 1996), it has been suggested that flies may be able to increase feeding rates to

compensate for reduced nutrient availability, and therefore that they may not be dietarily restricted when the food is diluted (Cooper *et al*., 2004). More recently, studies have provided evidence for and against compensation with some measurements showing that compensatory feeding does occur (Carvalho *et al*., 2005) and others that it does not (Mair *et al*., 2005), while further study suggested that flies subjected to DR lower their food intake (Min and Tatar, 2005). Clearly, some confusion exists and a reliable method to measure food intake in *Drosophila* is needed.

In mammals, such as mice, rats and primates, the amount of food ingested can be calculated by weighing the food before and after feeding. However, flies consume volumes of food that are too low to weigh accurately, and feed by extension of their proboscis into the food medium, prohibiting direct observation of the volume of food ingested. One method has overcome this problem by measuring the food consumed in liquid form in a capillary feeder (CAFE) (Ja *et al*., 2007). However, despite being effective for quantifying intake, CAFE feeding substantially reduces both egg-laying and lifespan compared to those seen in flies provided with food in the usual agar-gelled medium (Bass *et al*., 2007; Lee *et al*., 2008). This may be because in nature *Drosophila* feed on microorganisms, particularly yeast, on the surface of fruit (Carson, 1971; Kimura *et al*., 1977), and thus feeding on a liquid diet from a capillary may not reflect their natural food supply and feeding behaviour.

To overcome the problems of measuring food intake when flies feed on gelled media, several studies have made indirect measures of food uptake after marking the food, either with a visible dye (Edgecomb *et al*., 1994; Bross *et al*., 2005; Min and Tatar, 2005) or with radioactively-labelled nutrients (King and Wilson, 1955; Brummel *et al*., 2004; Carvalho *et al*., 2005). However, such 'tracer' methods have limitations and can even give misleading results. For instance, transferring flies to labelled food may change the food intake of flies such that immediate measurements may not reflect steady state feeding during undisturbed conditions. An alternative method involves observation of fly proboscis-extension onto the food surface, where the proportion of time with the proboscis extended is taken as a measure of food intake (Mair *et al*., 2005). A potential problem with this assay is that flies may alter the volume of food they consume per time with the proboscis extended, and thus the behavioural measure could provide an inaccurate representation of total food intake.

5.1.1. Problems with food labels to measure food intake

The food dye, F D & C blue 1, is a marker commonly used to label fly food in order to measure the volume of food consumed (Carvalho *et al*., 2005; Min and Tatar, 2005). The dye cannot be broken down or absorbed through the gut wall (Shimada *et al*., 1987). Flies are generally allowed to eat the dyed food for a fixed time and the corresponding volume of dye found in the fly is taken to reflect the volume of food eaten (Edgecomb *et al*., 1994; Carvalho *et al*., 2005). However, with time the volume of dye in the fly is controlled not only by the rate of ingestion of food (its input) but also by the passage time through the gut, which could vary, for instance with the level of nutrition in the food (Zanotto *et al*., 1993), the nutritional status of the animal (Raubenheimer and Simpson, 1994; Melcher and Pankratz, 2005) or its genotype (Ishimoto *et al*., 2000; Melcher and Pankratz, 2005). Thus, the total dye accumulated in the animal reflects the rate of food intake from when feeding on the dyed food starts until egestion of the marker commences.

The usage of radioactively labelled metabolites as markers of food uptake introduces further complications, because isotopes can pass through the gut wall into the body. The level of isotope absorbed is dependent upon the fly's nutritional requirements (Raubenheimer and Simpson, 1994), the ease with labelled isotopes of elements associate with compounds in the body (Carvalho *et al*., 2005; Geer *et al*., 1970; Thompson and Reeder, 1987), the capacity of the fly for the element, metabolic turnover rates and rates of excretion. Furthermore, removal of the radioactive tracer can also occur by egestion and/ or expulsion via egg production in females.

To illustrate the dynamics of label accumulation in a fly, first, data from a study which measured ^{14}C-choline labelled food intake by *Drosophila* was analysed[16] (Geer *et al*., 1970). The values obtained were then used to generate a simple model to illustrate a time course of food label accumulation:

(**Equation 5.1**)[17]

$$m(t) = -\left(\frac{c}{s}\right) \times \left[1 - \exp^{(s \times t)}\right]$$

[16] Analysis was performed by Matthew D.W. Piper using GraphClick (Arizona).
[17] Model generated by Eric Blanc.

The model was generated using three parameters: ingestion rate (*c*), rate of label removal (*e*) and the internal pool size in the fly (*p*). *m(t)* is the amount of labeled material inside the fly at time *t*, and *s* is the fraction of labeled material removed from the fly (*e* divided by *p*). The model was fitted through least-squares minimisation. (**Table 5.1** and **Figure 5.1.1**).

	Parameters		
Fly group	**Feeding rate (*c*)**	**Fraction of label**	**χ^2**
Non-reproducing females	53.689	- 0.053	3198
Reproducing females	108.237	- 0.109	2682
Non-reproducing males	13.344	- 0.016	411
Reproducing males	24.825	- 0.075	67

Table 5.1 Parameter estimates of ^{14}C-choline accumulation in female *Drosophila*.
s is obtained by dividing the rate of label removal (*e*) by the internal pool size in the fly (*p*). The parameters were obtained by least-squares fit displayed with the values of a chi-squared test of the data.

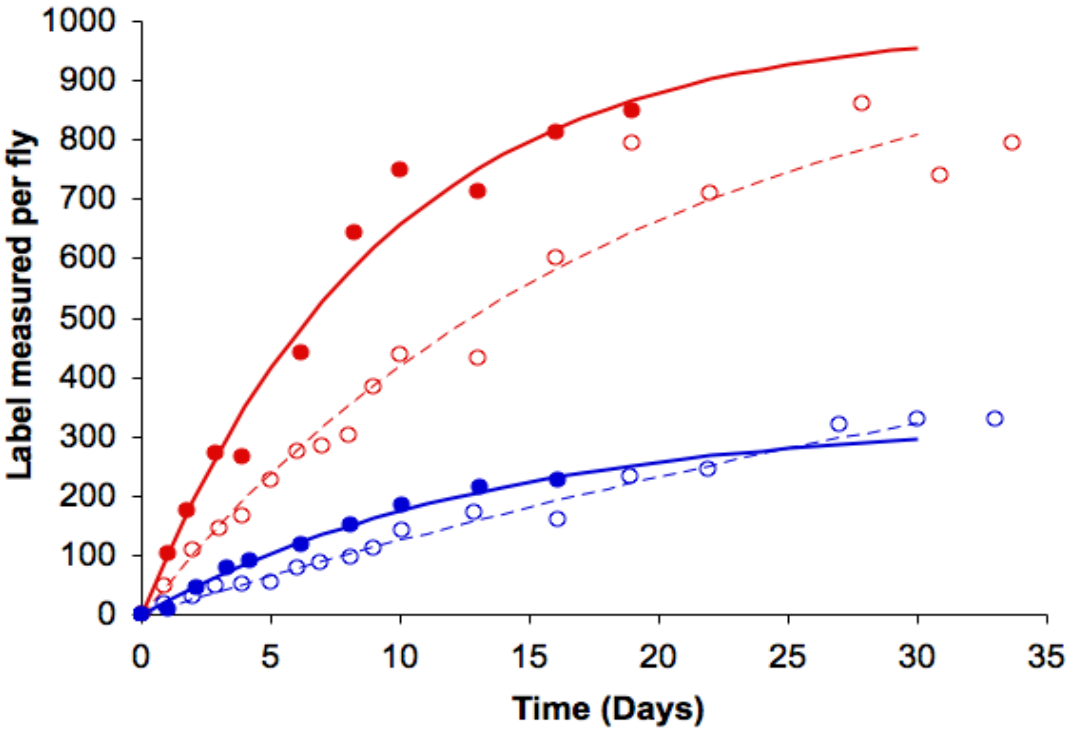

Figure 5.1.1 The accumulation of ^{14}C-choline in male and female flies.
The data was taken from Geer *et al.* (1970). The authors transferred reproducing and non-reproducing flies of both sexes onto labelled food at time 0 and measured label present at the various time points. The lines in the graph show the fit of the model given by Equation 5.1 to the data (circles). Reproducing females solid red line and circles, non-reproducing females dashed red line and open circles, reproducing males solid blue line and circles, non-reproducing males dashed blue line and open circles.

Using the model above, the effect on the dynamics of food label accumulation in the fly can be compared by assigning arbitrary values to the parameters (**Figure 5.1.2**). In the figure, at 'equilibrium', egestion (removal rate of the label) must equal its ingestion rate. However, flies can differ from each other by the rate of ingestion (and therefore also removal rate) but have equal internal pool sizes for the label, or alternatively, flies can have equal ingestion and removal rates but differ in internal pool size. By assigning arbitrary values to the parameters of the model, we can determine the effect of varying these parameters. The label accumulation profile (**Figure 5.1.2A**) can be categorised into 3 phases: an 'initial phase' in which the label is taken up in the food through feeding but before egestion has commenced, an 'intermediate phase' where label egestion has commenced but the label ingestion rate exceeds its egestion rate, and an 'equilibrium phase' in which the label egestion rate equals its intake rate. The fly with the higher feeding rate (green-labelled) accumulates label more rapidly than either of the other two flies in the initial phase but the amount of label plateaus at the same level as that for controls (blue-labelled). The fly with the higher internal capacity (red-labelled) shows the same rate of accumulation of label as the control fly in the initial phase, but its greater internal capacity means that the label enrichment is slower to reach equilibrium and accumulates to a higher level in the 'equilibrium phase' (**Figure 5.1.2A-B**). These findings show that the amount of label in the fly gives an accurate estimate of its feeding rate only during the initial phase, before egestion commences. During the intermediate phase, the label present in the fly will underestimate the feeding rate of a fly with higher ingestion rate than controls (green-labelled versus blue-labelled) and will then completely fail to detect it once the equilibrium phase is reached. For a fly with a greater internal capacity (red-labelled), the amount of food label relative to controls (blue-labelled) will consistently over-estimate the actual rate once egestion starts ('intermediate phase'), to an extent that reaches a maximum at the 'equilibrium phase' under steady state conditions.

Recent results obtained from label accumulation methods (Carvalho *et al*., 2005; Min and Tatar, 2005) have not addressed these issues, yet Carvalho *et al*. (2005) claim that compensation for food dilution occurs, while Min and Tatar (2005) claim that fully-fed flies significantly increase their food intake compared to DR flies . In order to assess whether DR flies do alter their food intake, I first investigated the rate of turnover of the

dye label in order to assess the stage when the label accumulation method becomes inaccurate, and then whether DR flies are indeed receiving less nutrition compared to fully-fed flies.

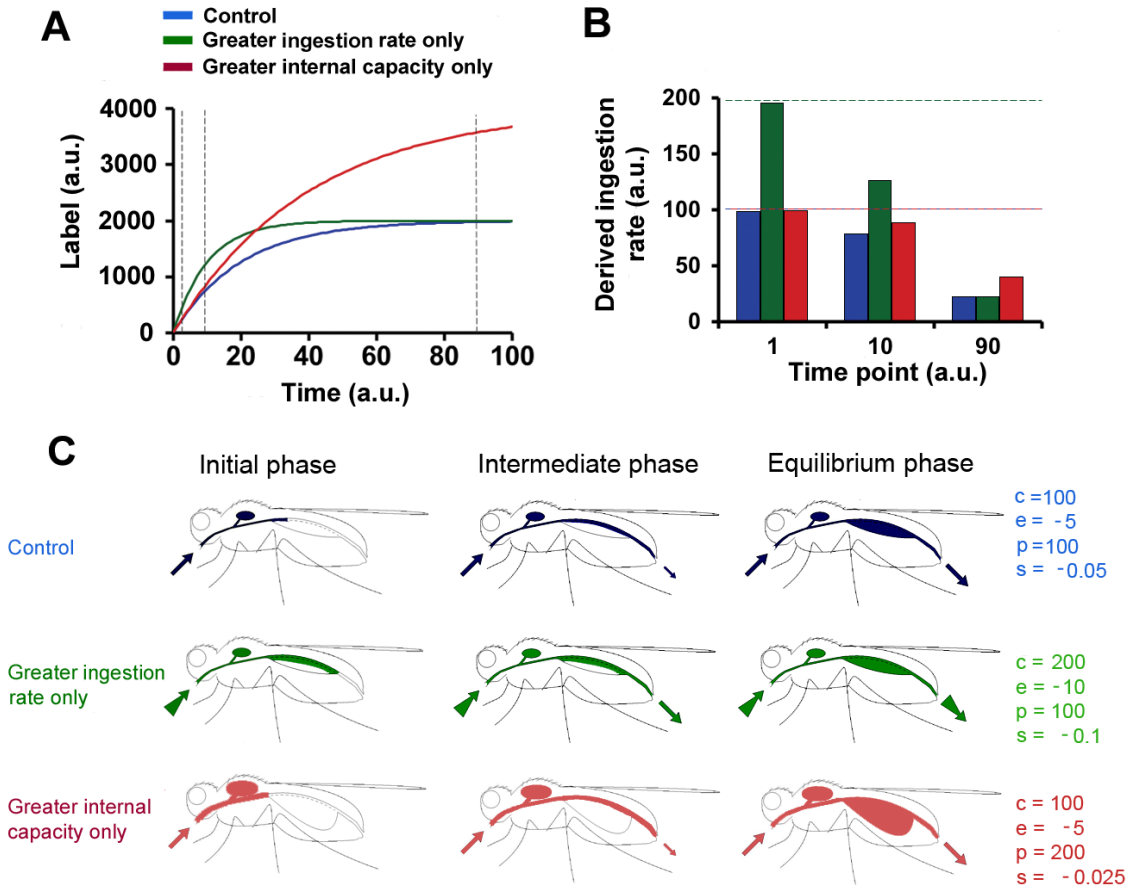

Figure 5.1.2 Characteristics of labelled food accumulation in flies.
(A) Modelled dynamics of accumulation of dye with time for the flies assigned arbitrary values of ingestion rate, *c*; internal capacity, *p*; and rate of label removal, *e*. **(B)** Ingestion rates for the three conditions modelled in A, derived by measuring the label accumulated at the indicated arbitrary times. If sampled during the 'initial phase' (*t* = 1 arbitrary unit), the observed ingestion rate reflects the real ingestion rate (dashed lines). During the 'intermediate phase' (*t* = 10 arbitrary units), the apparent ingestion rate is lower than the real rate and more so in the fly with the greater ingestion rate. In the 'equilibrium phase' (*t* = 90 arbitrary units) the apparent ingestion rate falsely gives the impression that flies represented in red have a higher ingestion rate than those represented in blue or green. At this point, the measurement only reflects the internal capacity of the fly for the label. **(C)** A pictorial representation of what may be occurring in the fly for each condition. The fraction of label material removed from the fly, *s*, is obtained by dividing *e* by *p*.

5.2. Methods

5.2.1. Fly stocks

Dahomey female flies were used in all experiments unless stated otherwise. The *chico*^{1} allele is maintained as a balanced stock that has been backcrossed to the Dahomey outbred laboratory population as described in Clancy *et al*. (2001). *sn*^{w}, *ry*^{506}, *to*^{1} (takeout) flies were a gift from Brigitte Dauwalder. All flies were maintained at 25^{o}C, 65% humidity, on a 12h: 12h light: dark cycle. Unless stated otherwise, all assays used mated females at day 7 after eclosion. Day 7 was chosen because the flies are still young, but several early adult developmental processes have been completed (Johnson and Butterworth, 1985). All flies were reared for assays at a standard density, as for lifespan studies (Kennington *et al*., 2001), and allowed to mate for 48h post emergence before being sorted by sex, under light CO_2 anaesthesia, into 30mL glass vials containing 7mL food.

5.2.2. Preparation of dye-labelled food medium

Food medium was prepared as described in **2.2.1**, but just before dispensing into vials FD & C No.1 blue dye (2.5% w/v) was added to the medium (**Figure 5.2.1**).

5.2.3. Measurement of gut passage time

Flies were reared and maintained as in a lifespan assay; and were fed throughout their lifetime and throughout the assay on either a full or DR diet. Flies were maintained in food bottles until 24h prior to the assay when they were sorted into 5 flies per vial. During the assay, flies were transferred to blue-dyed food for 15, 30 and 60 minutes and allowed to feed. Flies were then transferred back to non-coloured food and observed. The length of time required for first appearance of blue-coloured faeces was recorded for each condition.

5.2.4. Crop measurements

Females were dissected in phosphate buffer solution and placed onto a dimple slide. Crops were then photographed using a camera (Marlin F-145C2, Allied Vision Technologies) attached to a dissection microscope (Nikon C-DSD230, Japan) at 60x magnification. The surface area of the crops was measured double-blind using Object-Image v2.10 (imaging software by Norbert Vischer, University of Amsterdam) and the size calculation converted from an image of a 1mm^2 reticule.

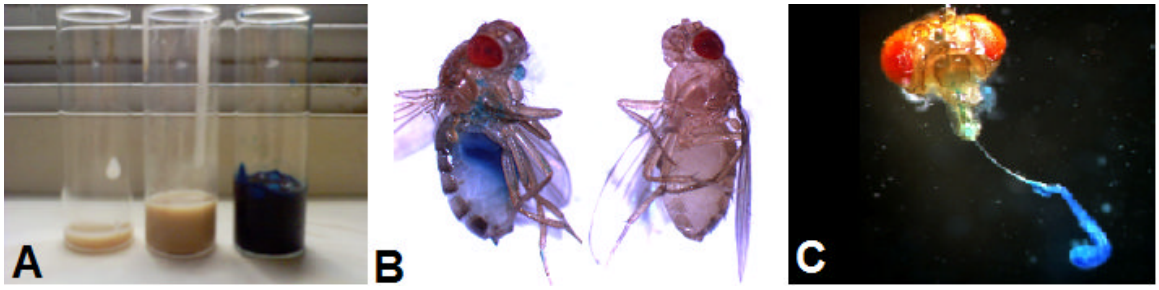

Figure 5.2.1 Blue dye, a visible marker for food consumed in the fly.
(A) Types of food used in blue spectroscopy assays. A vial containing 1ml of SY medium (left), a vial containing the normal volume (4ml) of SY medium (middle) and a vial containing SY medium with 2.5% (w/v) FD & C No.1 blue dye. **(B)** A female with labelled food in its digestive tract (left) and control female (right). (C) Head of a female with oesophagus and crop attached.

5.2.5. Colour spectrophotometry

Flies were homogenised in 200µL of distilled water. A further 800µL of distilled water was added and the suspension passed through a 0.22µM Millex filter (Millipore Corporation, Bedford) to remove debris and lipids. The absorbance of the liquid sample was then measured at 629nM [Hitachi U-2001 Spectrophotometer (Lambda Advanced Technology Ltd., UK)]. Age-matched flies exposed to non-dyed food were used as the baseline during spectrophotometry. The amount of labelled food in the fly was calculated from a standard curve made by serial dilution in water of a sample of blue food.

5.2.6. Proboscis-extension assay during undisturbed conditions

For undisturbed observations of feeding, 7-day-old mated flies of the same sex, were transferred to new food at a density of 5 per vial on the evening before the assay. Flies were maintained in a pooled population, 100 flies per bottle, and a subset was collected

and returned before and after the assay. Different measurements on different days were therefore considered to be independent of each other. Vials were coded and placed in a randomised order in rows on viewing racks at 25^{o}C overnight. The assay occurred with minimal noise and physical disturbance to the flies. To avoid recording disturbed fly feeding behaviour, 30 minutes was allowed between the arrival of the observer and commencement of the assay. Observations were performed 'blind' the next day for 90 minutes, commencing one hour after lights-on. In turn, each vial was observed for approximately 3 seconds during which the number of flies feeding was noted. A feeding event was scored when a fly had its proboscis extended and touching the food surface while performing a bobbing motion. Once all vials in the experiment had been scored in this way, successive rounds of observations were carried out in the same way for the whole 90 minutes of the assay, which, depending on the size of the experiment meant that each vial was observed once every 2 to 5 minutes. At the end of the assay, the vial labels were decoded and the feeding data expressed as a proportion by experimental group (sum of scored feeding events divided by total number of feeding opportunities, where total number of feeding opportunities = number of flies in vial x number of vials in the group x number of observations). For statistical analyses, comparisons between experimental groups were made on the totals of feeding events by all flies within a vial, to avoid pseudoreplication.

5.2.7. Combined proboscis-extension and blue dye assay

Groups of five 7-day-old mated flies were transferred onto fresh food medium as indicated containing 2.5% (w/v) blue food dye (F D & C Blue Dye no.1). Vials were scored approximately every 2 minutes for proboscis-extension and after a total of 30 minutes were transferred to eppendorf tubes and snap frozen in liquid nitrogen.

5.2.8. Statistical analysis

Statistical analyses were performed using R, v2.2.1 (R Development Core Team, 2005). To assess the relationship between proboscis-extensions and accumulation of blue dye, a linear mixed effects model was used. This modelled blue dye accumulation as a function

of proportion of time observed feeding. Genotype, age and food concentration were specified as fixed effects and trial date as a random effect. To test for non-linearity, a quadratic term of observed feeding events was added to some models. The model fit for the data was reasonably acceptable, judging from residual plots and qq-plots (per trial date). For thoroughness, we re-analysed all models on log-transformed data. Although this further improved the normality of the residuals, the conclusions of the models were qualitatively unaffected.

To compare the effect of time of day, group size and dietary composition on feeding frequency, we used generalised linear models (with binomial error structure and logit link function, the deviances were scaled to correct for over-dispersion, and using *F*-tests for analysing significance). The generalised linear models incorporate information on the sample sizes and use weighted regression analyses. Significance among factor levels (e.g. among the 4 different group sizes) was determined by model simplification, where we evaluated whether combining >1 factor level into a single level led to a significant increase in deviance of the model, using *F*-tests (Crawley, 2005). The same generalised linear models were also used to compare the proportions of time spent feeding in the combined assays.

5.3. Results

5.3.1. Selecting a suitable concentration of dye

Quantification of food intake by food labelling does not measure the food consumed, but the accumulation of the label dissolved in the food. It is thus important to investigate whether the label, F D & C Blue Dye No.1, has an effect on feeding behaviour, as it has been suggested that dyes act as a phagodeterrant in diluted food (Simpson and Raubenheimer, 2007). Dahomey female *D. melanogaster* were transferred to DR and full food containing 3 different dye concentrations for a 30-minute period, after which the amount of dye in the flies was measured with a colour spectrophotometer. The equivalent volume of food ingested can be calculated by measuring the wavelength of dye in known standards of dyed food. The amount of food consumed between females on 1% and 2.5% dyed food (P = 0.145, Wilcoxon rank sum test); and 2.5% and 5% dyed food

(*P* = 0.385) was not significantly different (**Figure 5.3.1**). There was also no significant difference between DR and fully fed flies at 1% (*P* = 0.394), 2.5% (*P* = 0.915) and 5% (*P* = 0.559) dye concentrations. However, lowered variations in readings were observed at lower concentrations of dye (1%), which suggests a lowered sensitivity in the spectrophotometer. Therefore, to maximise sensitivity and minimise potential interference with feeding behaviour, 2.5% dye concentration was selected as the concentration of dye to add to the food.

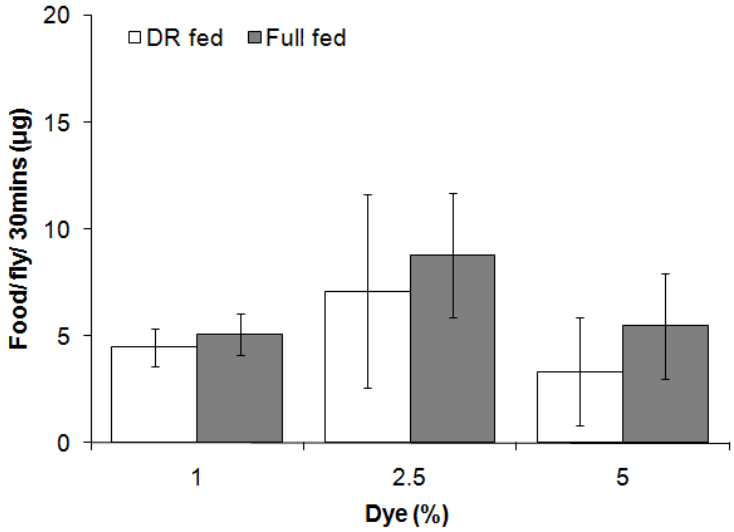

Figure 5.3.1 The effect of blue dye concentration and food intake. Flies were subjected to varying concentrations of blue-dye added to DR and full food for 30 minutes. The volume of food ingested during that period was not significantly different from each other. (*N* = 30).

5.3.2. The dynamics of the label accumulation in restricted and fully fed flies

As a fly begins to consume dyed food, the label accumulates at a rate that reflects the rate of its ingestion until the digestive tract becomes saturated with the dye and/ or egestion of the dye takes place. As the volume of dye within the animal approaches saturation, the rate of label accumulation decreases to a plateau (see **Figure 5.1.2**). If the label accumulated were measured at this time, it would reflect the gut capacity of the animal and not the rate of ingestion. Thus, it is important that the dynamics of the label accumulation is measured so that the various phases of accumulation are recorded[18].

Dahomey female flies were transferred to DR and full food containing dye and allowed to feed, after which experimental cohorts were sampled at 30, 90, 180, 360, 540 and 1440 minutes (**Figure 5.3.2A**). Initially, DR and fully-fed flies accumulated label at similar rates. However, rate of accumulation declined faster and label reached lower equilibrium

[18] These findings have been published in Wong *et al.* (2008) (see Appendix 3).

levels in fully-fed than in DR flies. Had dye accumulation reflected only ingestion rate, these results would imply that up to 30 minutes there was no ingestion rate difference between groups but that by 3 hours of feeding there was an approximately 1.5-fold higher ingestion rate in the DR flies. The data therefore suggested that flies under DR may alter the retention time of the food by having a larger gut capacity.

To investigate this possibility, both the passage time of food through the digestive tract and the size of the crop were measured in DR and fully-fed flies. The crop is a collapsible food storage sac that holds ingested food temporarily until it is ready to be passed on to the midgut. The rate at which the food passes through the crop is governed (via the central nervous system) by internal sensing mechanisms and stretch receptors in the abdomen and foregut (Edgecomb *et al.*, 1994). DR flies were found to have a 45% larger crop than fully-fed flies ($P < 0.0001$, Wilcoxon rank sum test) (**Figure 5.3.2B**). Furthermore, when flies were exposed to dye-labelled food for 15 or 30 minutes, dye took less than 50 min to start appearing in faeces (**Figure 5.3.2C**). There was no significant difference ($P = 0.7962$, Wilcoxon rank sum test) between passage times for DR and fully-fed flies. Thus, by 30 min, the amount of dye accumulated in the fly reflected feeding rate alone, while after 50 min it reflected the rate of label ingestion, the rate of egestion and the gut capacity. The measurements of crop size showed that the gut capacity was increased by DR.

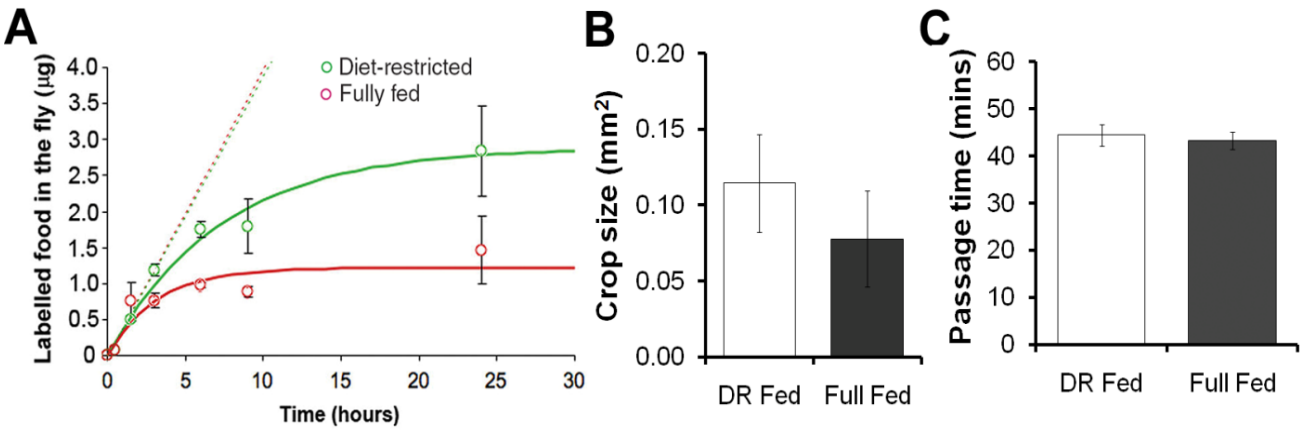

Figure 5.3.2 The differences in physiology between DR and fully fed flies can affect the dynamics of label accumulation.

(**A**) The amount of labelled food present in DR (green) and fully-fed (red) flies at different times after transfer from unlabelled food to food labelled with blue food-dye. Solid lines represent measured dye accumulation and dashed lines represent the label accumulation profile that would occur if feeding rates were the only factor governing label accumulation. Error bars = s.e.m. $N = 6$ for each timepoint. (**B**) The crop sizes of DR flies were significantly larger than the crop sizes of fully fed flies ($P < 0.0001$; $N = 50$). (**C**) The time blue labelled food takes to pass through the digestive tract of DR and fully fed flies is not significantly different ($P < 0.7962$; $N = 30$).

Thus, DR and fully-fed flies do not have a different ingestion rates if only the data from the first 30 minutes of the label accumulation experiment are considered (i.e. before any egestion takes place). However, this is a time period during which disturbance from the transfer of the flies may affect their feeding behaviour. There is thus a requirement for a method of measuring feeding in undisturbed, experimental conditions.

5.3.3. Measuring the volume of food intake per proboscis-extension[19]

Establishing a relationship between volume of food intake and proboscis-extension

In nature and in the laboratory, fruit flies feed on the food surface, by extending their proboscis into contact with the food and drawing it in. The proboscis-extension assay measures the proportion of time spent with the proboscis extended onto the food surface (referred to as the 'feeding frequency') (Mair *et al*., 2005). The number of observations of proboscis-extension was then expressed as a proportion of the total number of observations. This assay offers two advantages over the methods already considered: 1) repeated assays can be performed with the same flies through time because no flies are sacrificed for measurements, particularly valuable in the context of work on ageing; 2) the observations are undisturbed and can be made on flies housed on standard laboratory food, and could be extended to other culture conditions. However, one criticism of the assay is that flies may alter the volume of food intake per unit of time with the proboscis-extended (referred to as the 'ingestion ratio') and thus behavioural observations may not provide an accurate index of the total volume of food ingested. Clearly, the volume of food intake per time with the proboscis extended needs to be assessed for its consistency.

The proboscis-extension method was tested by measuring the volume of food ingested using the dye-label method in parallel with observing the proportion of time spent with proboscis extended. If, the volume ingested did vary per proboscis-extension then a linear relationship would not be observed between the volume of dye ingested and the time spent with the proboscis extended. The assay period was confined to the 30 minutes after transfer, because the dye is egested shortly after this length of time (**5.3.2**). Thus a 30-

[19] All experiments in this section were performed with the help of Matthew D.W. Piper

minute exposure period to blue-dyed food ensured that all dyed food eaten during the assay is retained in the fly gut and none was lost by egestion.

First of all, groups of 5, 7-day-old mated female flies were allowed to feed for 30 minutes on food labelled with blue dye while the proportion of time they spent with the proboscis extended was simultaneously observed. Flies were then sampled, and the amount of blue dye present quantified. The level of dye accumulated in the group was plotted against the proportion of proboscis-extensions observed in that group (**Figure 5.3.3A**). A strong positive linear relationship was found between the Volume of blue food accumulated and the proportion of feeding events Observed (V/O) ($P < 0.0001$, linear mixed effect model, LMEM)[20]. The gradient of this relationship represents the ingestion ratio of the flies, because it describes the volume of blue accumulated per proboscis-extension. To test for non-linearity (for example, saturation or acceleration in the V/O relationship), a quadratic term to the statistical model was added. This quadratic term was not significant ($P = 0.62$), indicating the V/O relationship is indeed linear over the timespan measured (**Table 5.1**). The linear relationship demonstrated that the proboscis-extension method is an accurate indicator of food intake in female *Drosophila* under these conditions.

Male vs. females

In order to see if the V/O relationship varied, the combined assay was repeated with 7-day-old mated males and females to test whether the sexes differed in ingestion ratio (gradient of the V/O relationship) (**Figure 5.3.3B**). The ingestion ratio was constant in males and in females, as both were found to have a significant V/O relationship ($P < 0.0001$, LMEM). The gradients of these relationships were not found to be significantly different ($P = 0.9871$), indicating that the ingestion ratio did not differ between the sexes. However, the intercept of the male relationship was significantly lower than that for females ($P < 0.001$) and suggested that males across all observations contained a lowered basal level of blue dye content than in females (**Table 5.2**). This could be due to differences in body size and/or body composition (e.g., proportions fat, muscles and reproductive tissues). As in the previous analysis, the quadratic term was not significant

[20] Statistical models were generated and statistical analysis was performed by Bregje Wertheim.

(P = 0.54), indicating that a linear V/O relationship exists. In spite of the sexes sharing the same ingestion ratio, females were found to have fed more than males over the 30-minute period because they spent a greater proportion of time with the proboscis extended (2.8-fold more on average) than males (P < 0.0001, generalised linear model, GLM). This suggested it is possible for flies to increase their food intake by feeding for more of the time, rather than by consuming in a greater volume per unit of time with the proboscis extended, and it is possible to detect such differences in food intake.

Assay	Fixed effects	*P*-value	V/O relationship		
			Coefficient	**Estimate**	**S.E.**
Dahomey females	Observation	< 0.0001	Intercept	7.91	2.50
(NF = 210, NV = 42, 5 trials)			Gradient	42.10	7.99
Dahomey males vs	Observation	< 0.0001	Intercept F	19.53	2.82
Dahomey females	Sex	< 0.001	Intercept M	12.83	1.80 [b]
(NF = 200, NV = 40, 4 trials)	Obs :Sex	not sig.	Gradient	25.81	8.58
Fully fed vs DR	Observation	< 0.001	Intercept	15.68	2.40
(NF = 75, NV = 15, 1 trial) [a]	Diet	not sig.	Gradient	62.25	16.11
	Obs: Diet	not sig.			
chico heterozygous vs	Observation	< 0.0001	Intercept	4.50	2.96
Dahomey control	Genotype	not sig.	Gradient	55.04	6.02
(NF = 90, NV = 18, 3 trials)	Obs: Genotype	not sig.			
takeout^{1} vs Canton-S	Observation	< 0.001	Intercept	1.29	1.25
(NF = 60, NV = 12, 1 trial) [a]	Genotype	not sig.	Gradient	62.01	15.78
	Obs: Genotype	not sig.			
ovo^{D} vs *white*^{Dahomey}	Observation	<0.0001	Intercept *ovo*^{D}	56.40	16.48
(NF = 200, NV = 40, 4 trials)	Genotype	<0.001	Intercept *w*^{Dah}	28.65	8.60 [b]
	Obs: Genotype	<0.0001	Gradient *ovo*^{D}	205.52	37.22
			Gradient *w*^{Dah}	14.46	8.94

Table 5.2 Estimates of the relationship between volume of food intake and observation during different conditions.

A linear relationship was tested between blue dye accumulations and feeding frequency using ANOVA in linear mixed effects model. The *P* value of the interaction terms is also displayed, which indicated whether the regression coefficients differ between comparative conditions (NF = no. of flies per condition and NV = no. of vials per condition). Obs = observation

[a)] These assays were not repeated on different trial dates. The statistical analysis was therefore only on fixed effects, i.e., a regression analysis.

[b)] These standard errors are for the differences in the intercepts.

Dietarily restricted flies vs. fully fed flies

This method was then extended to examine the effect of other factors that could determine the physiology and behaviour of feeding flies. The nutritional environment may be such a factor, and is particularly important in the context of DR experiments where dietary dilution is employed to restrict access to nutrients. The combined assay was performed with 7-day-old mated females that were either DR or fully fed (Bass *et al*., 2007) (**Figure 5.3.3C**). Flies on differing yeast concentrations did not alter the ingestion ratio, because no significant difference in V/O relationship was found ($P < 0.0001$, linear regression model), with no significant differences in the gradient or intercept of this relationship between the two different diet regimes (P =0.447, respectively, P = 0.304: **Table 5.2**). Flies on the DR diet were also found not to compensate for the reduced nutrient availability by feeding more often, because the proportion of proboscis-extensions between DR and fully fed flies during the 30-minute period of the combined assay were not different either (P = 0.3693, GLM).

Mutants with known or suspected differences in food intake:

chico^{1} *heterozygotes vs. Dahomey controls*

The first mutation, *chico*^{1}, is a null mutation in the single fly insulin receptor substrate in the insulin/insulin-like growth factor-1 signalling (IIS) pathway, a pathway suggested to affect foraging and feeding in larvae (Wu *et al*., 2005). The combined feeding assay was performed with 7-day-old mated female heterozygotes of *chico*^{1} and their genetic control (Dahomey) (**Figure 5.3.3D**). The ingestion ratio did not differ between *chico*^{1} heterozygotes and their controls, because a significant V/O relationship existed ($P <$ 0.0001, LMEM), with no significant differences in the gradient or intercept between *chico*^{1} heterozygotes and control flies (P = 0.3177, respectively, P = 0.3947, **Table 5.2**). *chico*^{1} heterozygous flies and their controls had the same food intake, because the proportion of proboscis-extensions between the cohorts during the 30-minute period of the combined assay were also not significantly different (P = 0.0831, GLM).

takeout^{1} *vs. Canton-S controls*

The second mutation, *takeout*^{1}, is in a gene reported to regulate the circadian rhythm and to increase food intake prior to starvation in *Drosophila* (Meunier *et al*., 2007). The

combined feeding assay was performed with 7-day-old mated *takeout*^{1} flies and their genetic control (Canton-S) (**Figure 5.3.3E**). The ingestion ratio did not differ between *takeout*^{1} flies and controls, because a significant V/O relationship existed ($P < 0.0001$, linear regression model) with gradient and intercept not significantly different between the two genotypes ($P = 0.5931$, respectively $P = 0.0549$: **Table 5.2**). This suggested that the ingestion ratios in *takeout*^{1} flies and controls were similar. However, *takeout*^{1} flies fed more than controls, because they spent 1.6-fold more time with their proboscis extended on the food than did Canton-S flies ($P < 0.05$, GLM). The flies thus elevated their nutrient-intake by feeding at a greater frequency, rather than by increasing the volume of intake per proboscis-extension.

ovo^{D1} *vs.* w^{Dahomey} *controls*

The final mutant studied, *ovo*^{D1}, causes female sterility and has been reported to induce a reduced feeding frequency (Barnes *et al*., 2008). The combined assay was performed with 7-day-old, mated, mutant females and their genetic control (*w*^{Dahomey}) (**Figure 1f**). A significant V/O relationship was found for both cohorts ($P < 0.0001$, LMEM); however, the gradient and the intercept for the relationship differed between the two genotypes ($P < 0.0001$ and $P < 0.001$, respectively). The V/O gradient for *ovo*^{D1} was steeper (205.52 versus 14.46 in *w*^{Dahomey}) and the intercept greater (56.40 versus 28.65 in *w*^{Dahomey}) than for *w*^{Dahomey} controls (**Table 5.2**). *ovo*^{D1} females thus ingested a greater volume of food per proboscis-extension compared to *w*^{Dahomey} controls (accumulated blue dye faster with each proboscis-extension). However, no significant difference in the proportion of time spent feeding between *ovo*^{D1} females and *w*^{Dahomey} controls was seen ($P = 0.6289$, GLM). This indicated that *ovo*^{D1} flies elevated their received nutrition by increasing the volume of intake per proboscis-extension rather than by feeding at a greater frequency.

Comparisons with flies of different ages

The effect of age upon the ingestion ratio was also analysed. Dahomey females were subjected to the combined blue dye and proboscis-extension assay at 4 different ages (day 7, 21, 35 and 50: **Figure 5.3.4.**). The V/O relationship was highly significant at all ages ($P < 0.0001$, linear regression model), but neither the gradient ($P = 0.0961$) nor the

intercept (P = 0.649) changed with age. The volume of intake per proboscis-extension was thus unaffected by the age of the flies. Additionally, there was no significant difference in the time spent with proboscis-extended over the 30-minute period at different ages (P < 0.6324, chi-squared test).

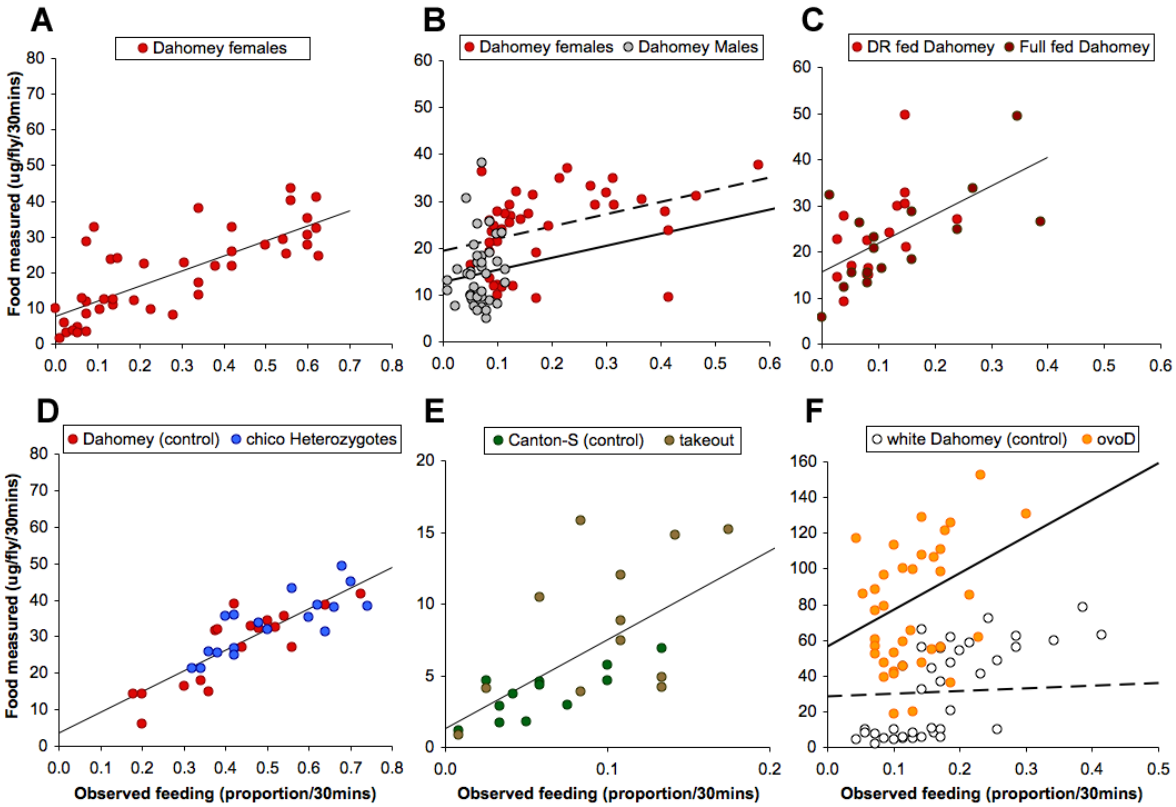

Figure 5.3.3 Measurements of blue label uptake after 30 minutes of feeding and the proportion of feeding events observed during this period.

One circle represents one vial containing 5 flies. Trend lines represent the relationship between the volume of food ingested and the observed proportion of flies feeding (V/O) described in **Table 5.2**. All flies were female unless stated, were 7 days old and were allowed to mate for 48 hours after eclosion (NF = the number of flies per condition, NV = the number of vials per condition). **(A)** A linear (V/O) relationship existed in mated Dahomey females (NF = 210, NV = 42). **(B)** The V/O relationships of mated Dahomey females (red circle, solid line) and males (grey circle, dashed line) did not differ significantly, although females were found to have fed at a greater frequency than males during the 30 minutes (NF = 200, NV = 40). The gradient for males did not differ significantly from that for females but had a lower intercept. **(C)** DR (red) and fully fed (dark red) Dahomey females shared the same V/O relationship and no difference in feeding between dietary conditions was found with the combined assay (NF = 75, NV = 15). **(D)** The V/O relationship was the same in *chico*^{1} heterozygotes (blue) and in the Dahomey control (red). No difference in feeding between genotypes was found with the combined assay (NF = 90, NV = 18). **(E)** The V/O relationship was the same in *takeout*^{1} (brown) and in Canton-S (green) females, even though *takeout* ^{1} flies were found to feed at a higher frequency than Canton-S controls (NF = 60, NV = 12). **(F)** Both *ovo*^{D1} (yellow, solid line) and *white*^{Dahomey} (white circle, dashed line) females had a positive V/O relationship, but *ovo*^{D1} flies had a significantly greater gradient and intercept, and therefore increased the volume of food ingested per proboscis-extension more quickly than *white*^{Dahomey} females (NF = 200, NV = 40).

During the 30 minutes of the combined assay, the flies consumed amounts of blue label that spanned a 30-fold range (equivalent to that found in 5µg – 150µg food). The food intake of the flies thus varied widely. Despite the variation in the overall amount of feeding, there was no significant variation in the ingestion ratios except in one genotype, *ovo*^{D1}. The variation in observed food intake is a possible indicator that transferring flies to labelled food may temporarily disturb their feeding behaviour and highlights the importance of measuring feeding during undisturbed conditions if a quantitative measure of normal intake is required. In addition, control-feeding frequency must be measured at the same time as that in the experimental treatments.

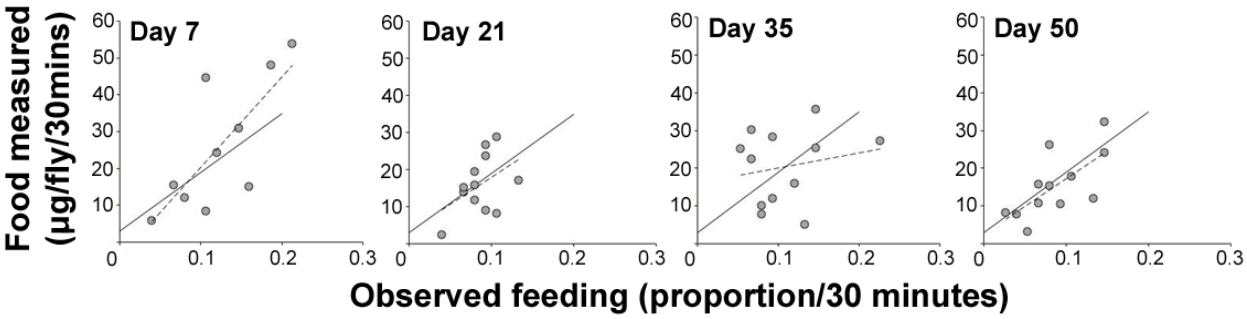

Figure 5.3.4 The ingestion ratio did not change for flies of different ages.
Circles represent measurements of blue label uptake after 30 minutes of feeding and the proportion of feeding events observed during this period. One circle represents one vial containing 5 flies. Experiments were conducted with mated Dahomey females. Assays occurred at 4 different ages: on days 7, 21, 35 and 50 after eclosion. Each assay used 60 flies (12 vials) that were taken from a population that began with 500 individuals. Solid lines represent the significant ($P < 0.0001$) V/O relationship with a gradient coefficient of 160.36 (S.E. = 31.39) and intercept of 2.89 (S.E. = 3.45), dashed lines represent the line of best fit for each age class.

5.3.4. Factors that influence feeding during undisturbed conditions

The proboscis-extension assay is a valuable method to compare undisturbed feeding between cohorts of flies. Having established in the previous section methods that demonstrated that the ingestion ratio remains constant for a number of conditions in *D. melanogaster*, it now becomes possible to investigate variables that could affect food intake during undisturbed conditions. These include the diurnal rhythm, which has been reported to alter feeding in *Drosophila* (Oishi *et al*., 2004), differences in group size, either in a positive (e.g. aggregation behaviour, Wertheim *et al*., 2006) or negative (e.g.

aggressive competition, Chen *et al*., 2002) direction, and finally, dietary composition may also affect feeding.

To test these factors, the undisturbed proboscis-extension assay was performed at 3 different times in the day. Flies were maintained in a 12h: 12h light: dark cycle with lights-on at 10am and lights-off at 10pm. The proboscis-extension assay was performed in the morning (at lights-on), in the afternoon (4 hours after lights-on), and in the evening (8 hours after lights-on) with 4 different group sizes (1, 2, 5 or 10 flies: **Figure 5.3.5A**). Both the time of day and the group size had highly significant effects on the proportion of time spent feeding ($P < 0.001$ for both group size and time of day, GLM), while the interaction between these two was not significant ($P = 0.88$). The lowest feeding proportion was observed in the morning for flies housed singly (0.15 of the time spent feeding), and this increased to approximately 0.50 in the afternoon and evening for flies feeding in groups of 5 or more. Both the afternoon and evening feeding proportions were significantly higher than those in the morning ($P < 0.0001$ in both cases, GLM). There was no significant difference in feeding proportions between flies during the afternoon and evening ($P = 0.182$, by model simplification). The lowest proportion of feeding was observed for flies housed singly 0.15 - 0.22 (depending on time of day), and this significantly increased to 0.18 - 0.31 (depending on time of day) when flies were housed in pairs ($P = 0.009$, GLM). The proportion of flies feeding was found to nearly double when the number of flies was increased to 5 per vial (0.32 - 0.49, depending on time of day; 2 flies per vial against 5 flies per vial, $P < 0.0001$, GLM), and did not increase further when flies were housed at 10 per vial (0.36 - 0.52, depending on time of day; 5 flies per vial against 10 flies per vial, $P = 0.287$, by model simplification).

Finally, the response of 7-day old female flies to two different yeast-based diets was measured, one diet made with water-soluble yeast extract (CSYExtract) (Carvalho *et al*., 2005) and the other with lyophilised yeast (SYBrewer's). The principle difference between these diets is that yeast extract contains only the water-soluble portion of an autolysed yeast culture, whereas the Brewer's yeast product is made of all cell contents and debris after autolysis and pasteurisation. Both of these are used to study the effects of DR (Partridge lab and Benzer lab) (**Figure 5.3.5B**). The foods 5x CSYExtract and 2x SYBrewer's represent fully fed (FF) conditions, while 1x CSYExtract and 1x SYBrewer's represent DR conditions. The food composition had a significant effect on feeding frequency ($P =$

0.0126, GLM). Flies exhibited a significantly lower feeding frequency when the concentration of yeast extract was increased in the CSYExtract diet (1x CSYExtract against 5x CSYExtract, $P = 0.0019$, GLM). In contrast, the feeding frequency of flies was unaffected when altering the yeast concentration of the SYBrewer's diet (1x SYBrewer's against 2x SYBrewer's, $P = 0.562$, GLM).

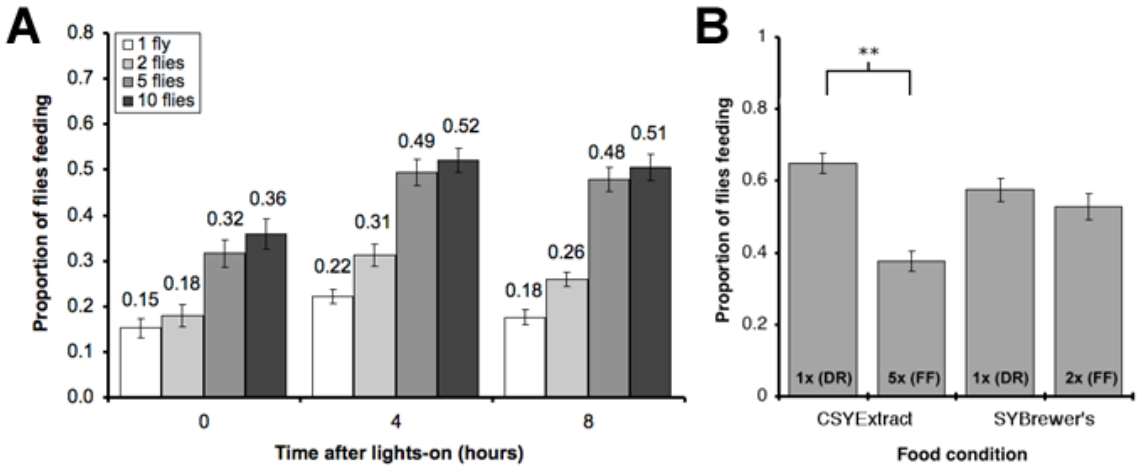

Figure 5.3.5 The possible factors that influence feeding frequency.
(A) The proportion of time spent feeding of 7-day old mated females over a 2-hour period at varying times after lights-on. Females were housed alone, or in groups of 2, 5 or 10 (the number of flies for each condition = 30, with 30 vials for single flies, 15 vials for groups of 2, 6 vials for groups of 5 and 3 vials for groups of 10). We found that increasing the number of flies per vial increased the feeding frequency of each fly, and overall, flies fed more frequently in the afternoon and evening. We calculated the proportion of time spent feeding by summing the scored feeding events divided by the total number of feeding opportunities, which is unaffected by the difference in the number of vials per condition. **(B)** The proportion of time spent feeding for flies fed different yeast-based diets. Flies were fed two similar diets containing either a water-soluble yeast extract (CSYExtract) or lyophilised yeast (SYBrewer's) at two different concentrations (DR = Dietary Restriction, FF = Fully fed). While feeding frequency was sensitive to the concentration of yeast extract in the diet, it was unchanged by the concentration of lyophilised yeast (NF = 60 and NV = 12 per condition: ** = $P < 0.005$, and error bars = S.E.).

5.3.5. Measuring food intake in lifespan studies

The proboscis-extension method allows repeated feeding assays to be performed with the same cohort of flies, an advantage over methods that sacrifice flies during measurements. No publication to date has studied either the feeding frequency of a cohort of flies throughout their lifespan or measured how much food flies consume throughout their lives. This is especially important when monitoring the effects of dietary restriction on lifespan, as the short-term probability of death as revealed by mortality analysis is rapidly affected by changes in nutritional conditions (Mair *et al.*, 2003). Thus

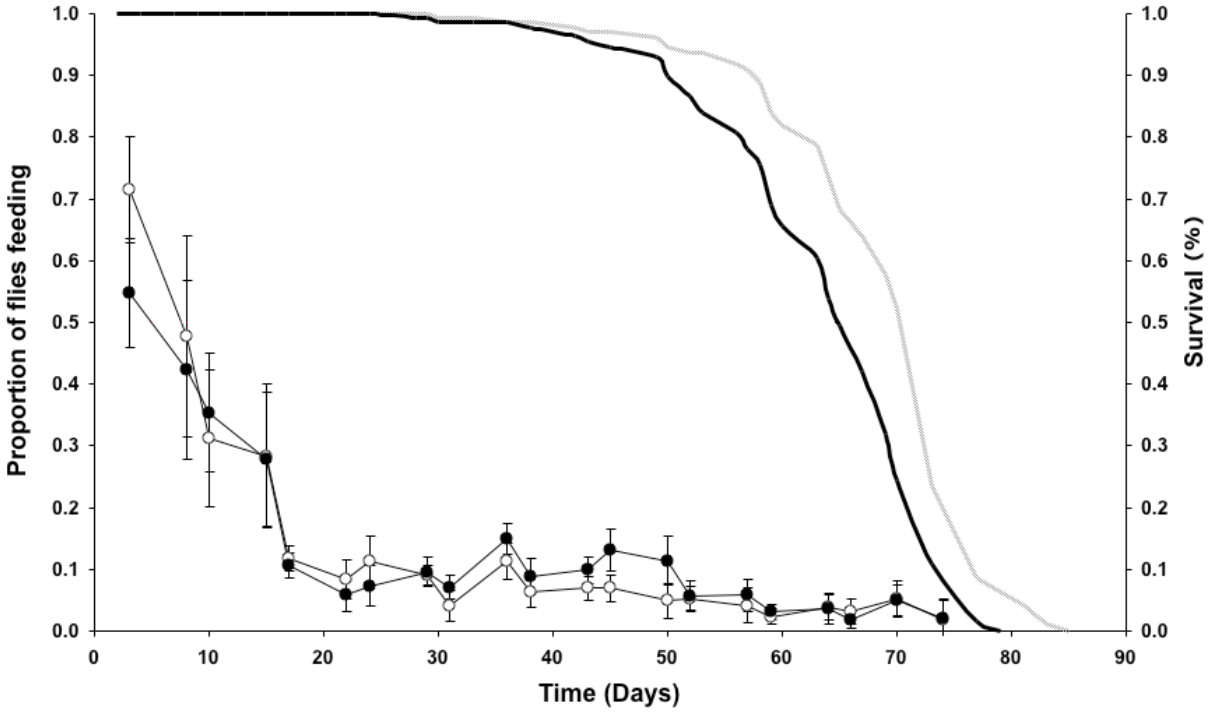

Figure 5.3.6 The proportion of time spent feeding in DR (open circles) and fully fed (FF) flies (closed circles) during their lifespan.
Survivorship curves are indicated with a solid grey line (DR) and a solid black line (FF) flies. Median lifespan: DR = 70 days, FF = 65 days. Proboscis-extension assays used 150 flies (30 vials) per condition. Flies were maintained in populations that began with 1500 individuals per condition (error bars = S.D.).

feeding data from a single time point early in life may not be informative about DR because they do not reflect nutrient intake changes that could occur close to the time of death.

The feeding frequency of once-mated females subjected to DR or control feeding was therefore compared over the course of their lifespan (**Figure 5.3.6**). The proboscis-extension assay was performed on cohorts of flies that were kept in a pooled population and assays were performed independently over their lifespan. Feeding declined markedly with the age of the flies, especially during the first 3 weeks of life. The changes in feeding frequency across the lifetime of the flies were significantly different on the two diets (significant interaction between Age and Diet, $P < 0.001$, GLM). No overall difference was found in average feeding frequency (0.17 in both cohorts) for the course of the lifespan. However, flies on a DR diet fed in a greater proportion of observations than fully fed flies early in life, while this reversed later in life when fully fed flies fed more than DR flies (between day 31 and day 50), after which the feeding became similar on the two diets.

Furthermore, experiments showed that the feeding frequency of flies on both diets were low at the beginning of the proboscis-extension assay but gradually increased to a steady state over 30 minutes (**Figure 5.3.7**).

The feeding frequency of wild-type and long-lived *chico*^{1} heterozygote flies (Clancy *et al*., 2001) was also compared over their lifespans[21]. Reduced *chico*^{1} signalling could lead to a reduction in food intake at some period of life, and therefore increased lifespan through self-imposed DR. Analysis of proboscis-extension over lifetime found that *chico*^{1} heterozygotes fed no more or less than Dahomey at any stage of their lifespan (P = 0.1639, GLM). Overall observed feeding proportions also did not differ significantly from wild-type controls (*chico*^{1} heterozygotes = 0.259 and Dahomey = 0.283, P = 0.3193, GLM: **Figure 5.3.8**). As observed before, feeding frequency declined markedly with the age of the flies for both genotypes, and this proved to be significant (P < 0.001, GLM).

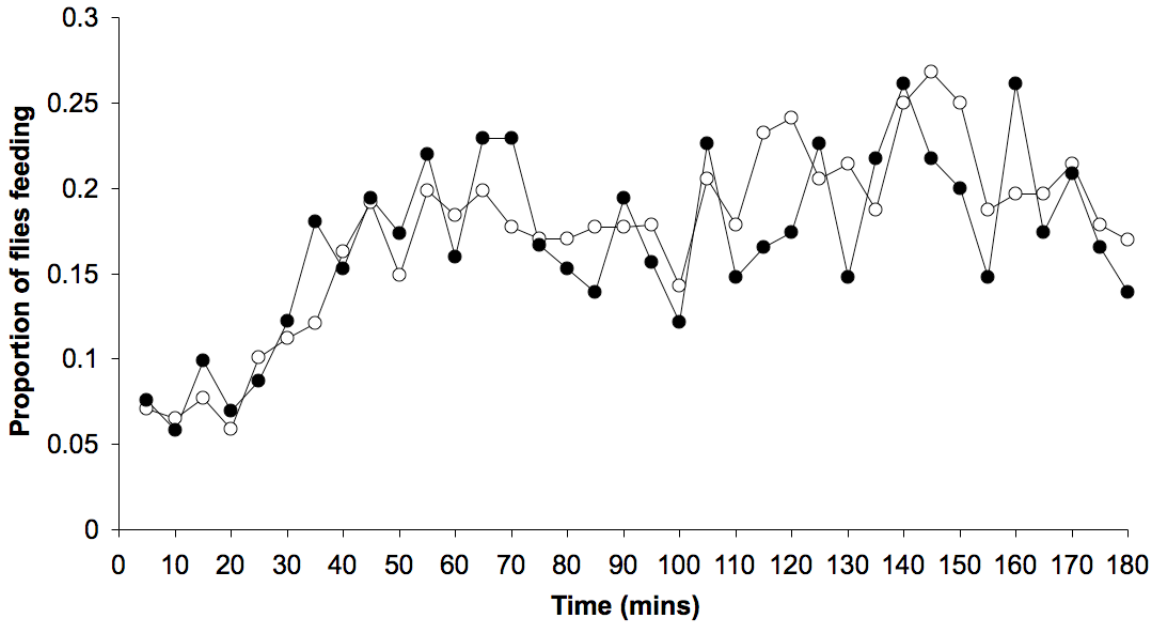

Figure 5.3.7 The proportion of time spent feeding during a proboscis-extension assay for DR (open circle) and fully fed (closed circle) once-mated 14-day old females.
Flies were maintained on different diets throughout their lifespan. DR females did not differ from fully fed females in feeding frequency. The assay began immediately when the observer arrived. Note the lower proportion of flies feeding during the first 30 minutes of the assay, which may relate to the appearance of the observer in the room (NF = 100; NV = 20).

[21] Performed with the help of Matthew D.W. Piper.

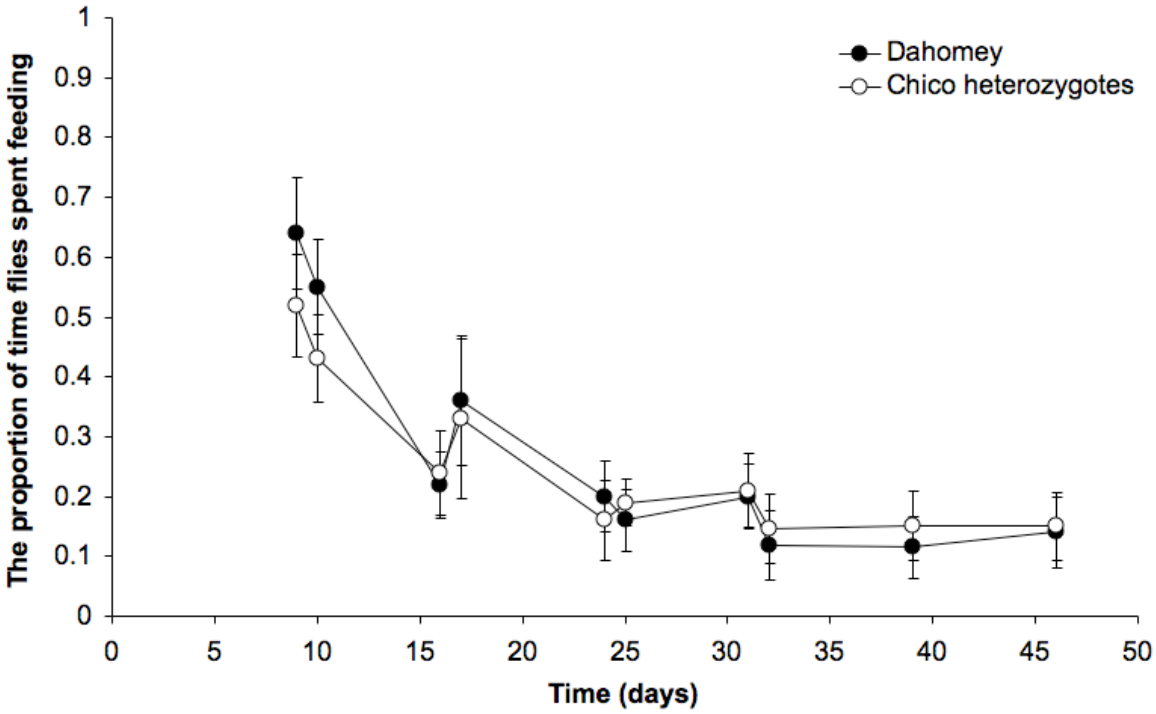

Figure 5.3.8 The observed proportion of time spent feeding for Dahomey (control) flies (closed circles) and *chico*[1] heterozygotes (open circles) on different days of their lifespan.
This experiment was performed with Matthew D.W. Piper, where observers alternately performed assays on the same population of flies. No significant interaction (P = 0.151) between the observers' data was found. Assays used 50 flies (10 vials) per condition, flies were maintained in populations that began with 500 individuals per condition; error bars = S.D.

5.4. Discussion

5.4.1. The proboscis-extension assay is a reliable indicator of food intake

In this chapter, the indirect method of assessing food intake in *Drosophila*, by observing proboscis-extensions, was validated by combining it with a direct method, measuring food intake with a food dye. Despite considerable variation in feeding between replicate groups of flies and between experiments performed on different days, the volume of food ingested per proboscis-extension (ingestion ratio) did not significantly differ between females and males, flies of different ages, flies subjected to DR and flies with mutations in *chico* or *takeout*. Only *ovo*^{D1} females ingested more dye per proboscis-extension.

Males fed less than females. The higher food intake of female flies is presumably related to their high nutrient-usage in egg-production (Lints and Soliman, 1988). The difference in intercept between the two sexes in the combined measurement indicates

that amounts of blue dye are always lower in males, although the increase in blue food per proboscis-extension is the same. These lower basal levels of dye may be due to the differences in size (the total volume of the crop and gut), or because the differences in body composition (e.g. fat tissue, vitellogenic material or muscles) may affect the spectrometer reading.

Sterile *ovo*^{D1} females exhibited a greater ingestion ratio than any of the other genotypes tested. This finding is surprising, because egg development is arrested in *ovo*^{D1} flies before the major nutrient investment occurs (Oliver *et al*., 1987). If the larger volume of food ingested reflects greater nutrient absorption and utilization in *ovo*^{D1} flies, it could be that they expend more energy through a higher level of activity than fertile flies. This could partially explain why a higher ingestion ratio was found in these mutant flies. *ovo*^{D1} has been reported as feeding less frequently during long-term undisturbed conditions (Barnes *et al*., 2008). The findings reported here may not contradict those of Barnes *et al*. (2008), because our data were obtained from the first 30 minutes after transferring to blue-labelled food, and no differences with feeding frequency was found, only with ingestion ratio.

The combined assay is not suitable for long-term, undisturbed feeding experiments because the assay requires that flies be transferred to dyed food, which disturbs fly feeding behaviour. Less proboscis-extension was seen than in steady-state conditions, and it only reached a constant level by the end of the 30-minute observation period. However, the indirect method alone is accurate for measuring fly feeding during long-term, undisturbed, experimental conditions once assessed by the combined assay for any differences in the ingestion ratio.

Indeed, the method provided evidence that flies exhibit marked diurnal differences in feeding behavior and a preference for feeding in groups. The effect of fly group size may reflect the role of aggregation pheromones, which act as communication signals between flies on breeding substrates, with feeding and oviposition rates increasing with the level of aggregation pheromone (Wertheim *et al*., 2006). The outcome of such studies may be more difficult to achieve or less accurate without a method that measures undisturbed steady-state feeding behaviour.

5.4.2. Flies subjected to dietary restriction do not compensate with increased feeding rate

DR in flies can be imposed by dilution of their food source, which is available in excess. Flies could therefore adjust their food intake to compensate for the reduction in nutritional value, thus reducing or eliminating the effect of food dilution on nutrient-intake. The literature on this topic is conflicting, with some reports that flies do and do not partially compensate for the food dilution (Carvalho *et al*., 2005; Mair *et al*., 2005) and others that even report increased food intake with increased nutrition (Min and Tatar, 2005). In this chapter, the labelled food method (Carvalho *et al*., 2005) was assessed and the dynamics of label accumulation was characterised. Given that these methods rely on measuring only the volume of label present in the fly, the results can be influenced by factors other than feeding. For instance, if the internal capacity of the flies for the label is increased by the experimental treatment, with no alteration in feeding then, with increasing times of exposure to the labelled food, the group with the higher internal capacity will give the spurious appearance of having a higher food intake. Here, flies subjected to DR were noted to have an increased internal capacity for food but their food intake rate was unchanged. This suggests that the greater volume of labelled food measured in DR flies reported in Carvalho *et al*. (2005) in fact reflected the internal capacity rather than the rate of food intake. Indeed, for the amount of label in the fly to reflect feeding, measurements must be confined to the time period before label egestion commences, about 40 minutes, a time period during which disturbance created by the transfer of flies affects their feeding behaviour. Thus, measurement of food intake of flies under DR is better performed with the proboscis-extension assay.

The yeast component of the fly diet was highlighted in chapter 3 to have an effect on the fly DR response. Various studies have reported the effects of DR (Carvalho *et al*., 2005; Min and Tatar, 2005), but they did not employ the same dietary conditions as each other. Therefore the yeast component of the diet was tested to see whether it could alter the feeding response to nutrient dilution, by comparing the effects on feeding frequency of DR using SYBrewer's yeast diet with that of a diet used in another published study, CSYExtract (Carvalho *et al*., 2005). Similar to the data reported by Carvalho *et al.* (2005), we saw feeding frequency decrease as the concentration of CSYExtract in the medium

was increased. In contrast, but consistent with previous reports (Mair *et al*., 2005), flies feeding on the SYBrewer's diet under DR and fully fed conditions did not change their feeding frequency. These data demonstrate that different DR recipes can elicit different behavioural responses. This is interesting because it may also mean that different diets affect lifespan-extension in different ways. The flies on SYBrewer's diet fed at the same frequency as flies subjected to DR conditions using CSYExtract, which suggests that flies on the fully fed CSYExtract diet decrease their feeding to avoid higher concentrations of food. This is consistent with yeast extract having a toxic effect on flies and shortening lifespan (**Chapter 3**). Furthermore, this may explain the difference in food intake between DR and fully-fed flies reported in Carvalho *et al*. (2005), where they wrongly concluded that DR flies elevate their feeding in response to lowered nutrition of the food, when in fact the fully fed flies decreased their feeding in response to the increasing toxicity of the food.

An important element of studies into ageing is the longitudinal effects of lifespan-altering interventions. Although, flies subjected to DR do not alter their feeding frequency on day 7 of adult life, it is still possible, however, that they do so later in life (day 40 onwards). Thus, a longitudinal study of feeding frequency under DR was conducted. Very early in adult life (day 3) DR flies exhibited a higher feeding frequency than those under fully fed conditions, but this did not occur over the majority of life and there were even individual instances of higher feeding frequency in fully fed flies (later in life) than those subject to DR. This agrees with a previous longitudinal data on feeding frequency under DR (Mair *et al*., 2005). This demonstrates that reduced nutrient intake does indeed correlate with extended lifespan for flies. Furthermore, it also shows that the level of food consumption in older flies is remarkably lower in comparison to feeding levels in early-life (up to day 14), and more experiments will be required to understand how this lowered nutritional intake may contribute to declining mortality rates observed in very late-life (Curtsinger *et al*., 1992).

Despite the accuracy of the proboscis-extension assay to measure the food intake of flies, it does not provide information on the level of nutrition being utilised. The data suggests that flies under DR do not increase their food intake but have adapted their gut capacity for increased absorption of food. It is well known that increased size of the digestive tract results in an increased digestive capacity in herbivores (Demment and van

Soest, 1985), and increases in gut size occurs as a result of reduction in food quality in birds (Kehoe *et al*., 1987; Brugger, 1992) and mammals (Parra, 1978; Sibly, 1981; Clauss *et al*., 2003) as well as insects (Yang and Joern, 1994b). Additionally, post-ingestive responses have been reported in *Locusta migratoria* that are fed varied concentrations of carbohydrate and/ or protein in their diet, where specific retention of the limited nutrient enables the organism to reach their maximal growth (Zanotto *et al*., 1993). Such digestive adaptations may indicate that DR flies extract relatively more protein and carbohydrate from the diet during absorption than do fully fed females. This indicates that accurate measurement of feeding intake (nutrition acquisition) may not reflect nutrient assimilation. While informative about the whole-food feeding rate, the proboscis-extension assay described here would only provide information regarding the behavioural feeding response in *Drosophila*. This can be useful for drug uptake studies and provide information regarding behavioural responses to DR and IIS manipulation.

5.4.3. Mutations in the IIS pathway do not alter food intake in flies

Mutations in the IIS pathway have been shown to extend the healthy lifespan of the nematode worm *Caenorhabditis elegans*, as well as *Drosophila* and the mouse (Partridge and Gems, 2002; Liang *et al*., 2003; Kenyon, 2005; Giannakou and Partridge, 2007). Hence, there is intense interest in understanding how the effects of this pathway on healthy lifespan are mediated. A possible cause for the lifespan-extension effect in flies is that they reduce their food intake, resulting in self-imposed DR. If true, this could also account for the observed overlap between the effects of altered IIS and DR in *Drosophila* (Partridge *et al*., 2005a). Null mutation of the gene encoding the insulin receptor substrate *chico* in *Drosophila* both extends lifespan (Clancy *et al*., 2001) and alters the response to DR (Clancy *et al*., 2002). It is therefore a good candidate to test whether flies have altered feeding. The ingestion ratio of long-lived c*hico* heterozygotes was not significantly different from controls, which suggests that *chico* does not consume a greater volume per proboscis-extension. Additionally, undisturbed, long term feeding frequency using the proboscis-extension assay found total food intake was not reduced in the mutants either. The increased survival of $chico^{1}$ mutant flies compared to controls can therefore not be explained by a reduction in food intake (Clancy *et al*., 2001). Thus the

observed extension in lifespan in *chico*^{1} mutants is not simply due to self-imposed DR (Mair *et al*., 2003).

Chapter 6: The role of dAKT in *Drosophila* lifespan

Abstract

The insulin/insulin-like growth factor-1 signalling (IIS) pathway controls growth, metabolism, lifespan and fecundity in diverse animals, including the fruit fly Drosophila melanogaster*. A major component of* Drosophila *IIS, the protein kinase AKT, has yet to be established as a regulator of lifespan, fecundity and stress resistance, even though strong evidence exists for a role of the genes upstream and downstream. In this chapter, experiments were performed to show that* dAkt *hypomorphs with an appropriately reduced level of dAKT activity have reduced size and fecundity and increased storage of metabolites such as trehalose, lipids and glycogen. Furthermore,* dAkt *mutants also have a 35% increase in median adult lifespan as well as resistance to oxidative stress. These findings indicate that the lifespan extension associated with IIS reduction is indeed mediated via AKT.*

6.1. Introduction

6.1.1. The role of Akt signalling in the insulin/IGF-1 pathway

In mammals, insulin and insulin-like growth factor (IGF) signalling control blood glucose metabolism, growth, stress resistance, reproduction and ageing (Tatar *et al*., 2003; Piper *et al*., 2008). Five types of dimeric insulin/IGF receptors are produced in mammals by combinations of monomers of a single insulin receptor and two IGF-receptor subtypes (Taguchi and White, 2008). Upon activation by ligand binding, a phosphorylation cascade occurs by associating insulin receptor substrates (IRS) 1 and 2 with phosphoinositide-3-OH kinase (PI3K) subunits p85 and p110, resulting in phosphatidylinositol (3,4,5)-triphosphate (PIP_3) production (White, 1998). PIP_3 is responsible for binding Akt to the plasma membrane via its pleckstrin homology (PH) domain, inducing a conformational change to allow phosphorylation and activation by pyruvate dehydrogenase kinase 1 and 2 (PDK1 and PDK2) (reviewed and references in Sale and Sale, 2008). From here, it is known to continue the cascade by phosphorylating a number of downstream targets.

Three major isoforms of Akt exist in mammalian cells: Akt1, Akt2, Akt3 (a.k.a. PKBα, PKBβ and PKBγ, respectively) (Jones *et al*., 1991; Altomare *et al*., 1995; Brodbeck *et al*., 1999; Galetic *et al*., 1999; Nakatani *et al*., 1999). The roles of Akt in insulin signalling are diverse, with each mammalian isoform contributing different effects. Akt1 mainly controls growth and adipogenesis (Chen *et al*., 2001; Cho *et al*., 2001), Akt2 has a key role in glucose metabolism, from increasing glucose uptake (Whiteman *et al*., 2002) to stimulating glycogen synthesis by glycogen synthase kinase (GSK-3a and GSK-3b) phosphorylation (Jiang *et al*., 2003; Katome *et al*., 2003), while Akt3 is responsible for brain size but not growth or glucose uptake (Easton *et al*., 2005). Mammalian Akt is known to signal protein translation via mammalian target of rapamycin (mTOR) by phosphorylation inactivation of tuberous sclerosis complex-2 (TSC2) (Inoki *et al*., 2002). Additionally, Akt is known to phosphorylate all four FOrkheard boX subgroup O (FOXO) family members: FOXO1 (FKHR), FOXO3a (FKHRL1), FOXO4 (AFX) and FOXO6, translocating them from the nucleus to the cytoplasm (Biggs *et al*., 1999; Brunet *et al*., 1999; Kops *et al*., 1999; Wolfrum *et al*., 2003). These FOXO factors have been found to target genes involved in differentiation, apoptosis, detoxification, metabolism, DNA repair and stress resistance, factors which are important in promoting longevity (Greer and Brunet, 2005). Thus, the Akts are antagonists to these processes. Indeed, lifespan extension has been reported in IGF-1R mutants (Holzenberger *et al*., 2003), adipose tissue specific IR knockout mice (Bluher *et al*., 2003) and IRS1 deletion mutants (Selman *et al*., 2008), suggesting the IIS pathway may regulate lifespan in mammals via FOXO.

In *C.elegans*, mutations in the insulin/IGF-I receptor homologue *daf-2* (Kimura *et al*., 1997) or in the PI3K homologue *age-1* (Morris *et al*., 1996) cause animals to arrest as dauers, shift metabolism to fat storage, and live longer (Kenyon *et al*., 1993; Larsen *et al*., 1995). Furthermore, mutations in the fork head transcription factor DAF-16, the homologue of mammalian FOXO, completely suppress the dauer arrest, metabolic shift, and longevity phenotypes of *daf-2* and *age-1* mutants (Kenyon *et al*., 1993; Larsen *et al*., 1995; Lin *et al*., 1997; Ogg *et al*., 1997), indicating that DAF-16, like in mammals, is a negatively regulated downstream target of *C. elegans* insulin receptor-like signalling. Two *C.elegans* homologues of the mammalian Akt family have been identified to antagonise DAF-16, AKT-1 and AKT-2, which also inhibit dauer arrest and longevity (Paradis and Ruvkun, 1998), suggesting the pathway is evolutionarily conserved.

6.1.2. The *Drosophila* IIS pathway and growth

In the fruit fly *Drosophila melanogaster*, the functions of the two mammalian signalling pathways (insulin and IGF-1) are united as insulin/insulin-like growth factor-like signalling (IIS) (**Figure 6.1.1**) (Giannakou and Partridge, 2007). Studies of the insulin-like receptor *InR* (the *insulin/IGF receptor* and *daf-2* homologue) (Chen *et al*., 1996) and *Dp110* (the *age-1* and *PI3K* catalytic subunit equivalent) (Leevers *et al*., 1996) have shown that the fly IIS pathway regulates imaginal disc growth and cell size. Thus increased IIS leads to increased growth and cell size, and decreased IIS leads to reduced growth and cell size. Growth in an organism can be the result of both cell proliferation and cell size, and mutations that inhibit IIS during development can affect both. For example, null mutations in *chico* (the InR substrate gene) and *dS6-kinase* (a target of the IIS pathway in mammals) slow imaginal disc growth development, eventually giving rise to viable dwarf adults with lower cell number and cell size (Bohni *et al*., 1999; Montagne *et al*., 1999). Null mutations of other identified components of the IIS pathway are lethal. However their function in imaginal disc and whole animal development has been investigated by ectopically expressing transgenes, analysing mutant clones, or using weak loss-of-function mutations. For example, flies with heteroallelic combinations of weak insulin receptor (*dInR*) alleles (Chen *et al*., 1996; Brogiolo *et al*., 2001) or flies homozygous for hypomorphic *dAkt* alleles (Stocker *et al*., 2002) are small and solely as a result of decreased cell size.

The completed *Drosophila* genome sequence has revealed the presence of at least seven *Drosophila* insulin-like peptides (DILPs), each of which can promote larval growth. In both larvae and adults, DILP2, DILP3 and DILP5 are synthesized in a bilaterally symmetric cluster of cells within the brain, known as the median neurosecretory cells (MNCs) (Brogiolo *et al*., 2001; Ikeya *et al*., 2002; Rulifson *et al*., 2002; Broughton *et al*., 2005). Significantly, ablation of MNCs causes developmental delay and growth retardation, an outcome similar to *InR* mutants. Ubiquitous expression of a DILP2 transgene rescues this phenotype (Rulifson *et al*., 2002), while ectopic expression of DILP2 produces large flies with increased cell size (Brogiolo *et al*., 2001). These data suggests DILPs function as ligands for dINR.

The dINR signals positive growth regulation and transmits at least part of its signal through CHICO, the insulin receptor substrate, and Dp110, the catalytic subunit of phosphatidyl inositol 3-OH-kinase (PI3K) (Leevers *et al*., 1996; Bohni *et al*., 1999). Indeed, disruption of Dp110 during eye and wing development also reduces cell proliferation and cell size (Weinkove *et al*., 1999). Furthermore, DPTEN was found to antagonise the effects of Dp110 on growth, consistent with the ability of PTEN to dephosphorylate phosphoinositides generated by PI3Ks. Thus, loss-of-function mutations in DPTEN increase cell size whereas over-expression of DPTEN generates small flies with small cells (Goberdhan *et al*., 1999; Huang *et al*., 1999; Gao *et al*., 2000).

Interestingly, the increase in cell size associated with loss of DPTEN function is suppressed in a background with reduced dAKT activity (Gao *et al*., 2000). Furthermore, a mutation in the PH domain of dAKT reduces its affinity for PIP_3 and is sufficient to rescue the lethality of flies devoid of DPTEN activity. Thus, PI3K is responsible for increasing levels of PIP_3, secondary lipid messengers that localise dAKT via its PH domain to the plasma membrane. At the plasma membrane, dAKT is activated by PDK-1 (Rintelen *et al*., 2001), and consequently enables it to phosphorylate a number of downstream targets related to growth (Stocker *et al*., 2002).

One phosphorylation target of dAKT is the dTSC-2 protein (Potter *et al*., 2002), which when targeted, results in the increase of dTOR signalling, and ultimately activation of S6 kinase, an effector involved in protein translation (Lizcano *et al*., 2003; Dong and Pan, 2004). Another target of dAKT is the nuclear forkhead transcription factor, dFOXO, the equivalent of nematode DAF-16 and the mammalian FOXOs (Junger *et al*., 2003; Puig *et al*., 2003), which is implicated in activating a growth suppressing eukaryotic initiation factor 4 binding protein (4E-BP). When dFOXO is phosphorylated by dAKT, it is no longer transported into the nucleus and is thus unable to promote 4E-BP and factors that increase longevity.

In the fly, mutational inactivation of either *dTOR* or the loss of S6K function results in a reduction of cell size but not cell number (Montagne *et al*., 1999), whereas over-expression of dFOXO increases levels of 4E-BP and leads to a reduction of cell number but not cell size (Junger *et al*., 2003; Puig *et al*., 2003). This suggests that dAKT activates growth by the inhibition of two separate pathways, inhibiting dFOXO for cell proliferation and inhibiting dTSC2 for cell growth. However, dTOR is also known to phosphorylate and

inactivate 4E-BP (Teleman *et al*., 2005), meaning dAKT may inhibit 4E-BP via both pathways. Indeed, it is suggested that flies with viable mutations encoding *dAkt* could either have a normal number of cells that are smaller in size (Verdu *et al*., 1999), or have a lower cell number and lower cell size (Bohni *et al*., 1999).

6.1.3. The *Drosophila* IIS pathway and lifespan

The majority of the literature on IIS signalling in *Drosophila* relates to growth and development in pre-adults, and its role in lifespan extension of adults is less well understood. However, manipulating genes singularly in the pathway does affect lifespan, stress resistance, metabolism and in some cases fecundity (reviewed in Giannakou and Partridge, 2007). Loss of function mutations in *dInR* (Tatar *et al*., 2001), or null mutation of *chico* (Clancy *et al*., 2001) results in dwarf flies with a longer lifespan, greater stress resistance and lower fecundity. Ablation of the MNCs at a late stage of development lowers DILP production, which extends lifespan, reduces fecundity and increases stress resistance, while body size is only marginally reduced (Broughton *et al*., 2005). These long-lived flies also have high levels of triglycerides (Bohni *et al*., 1999; Tatar *et al*., 2001), trehalose and glycogen (Broughton *et al*., 2005).

Importantly, lifespan extension has been uncoupled from reduced growth and fecundity in IIS mutants. dFOXO is known to be phosphorylated by the IIS pathway preventing it from activating longevity gene targets in the nucleus, and when over-expressed in the head fat body extends adult lifespan without reducing fecundity and body size (Hwangbo *et al*., 2004). Additionally, over-expression of *dFOXO* in the gut and abdominal fat body was sufficient in extending lifespan without reducing body size or fecundity (Giannakou *et al*., 2004; Giannakou *et al*., 2007). This finding suggests that the decreased fecundity described in other IIS models in *Drosophila*, such as $chico^{1}$ mutants (Clancy *et al*., 2001), *dInR* transheterozygotes (Tatar *et al*., 2001) and MNC-ablated flies (Broughton *et al*., 2005) is either not controlled via fat body *dFOXO* or is regulated by altered IIS earlier in life.

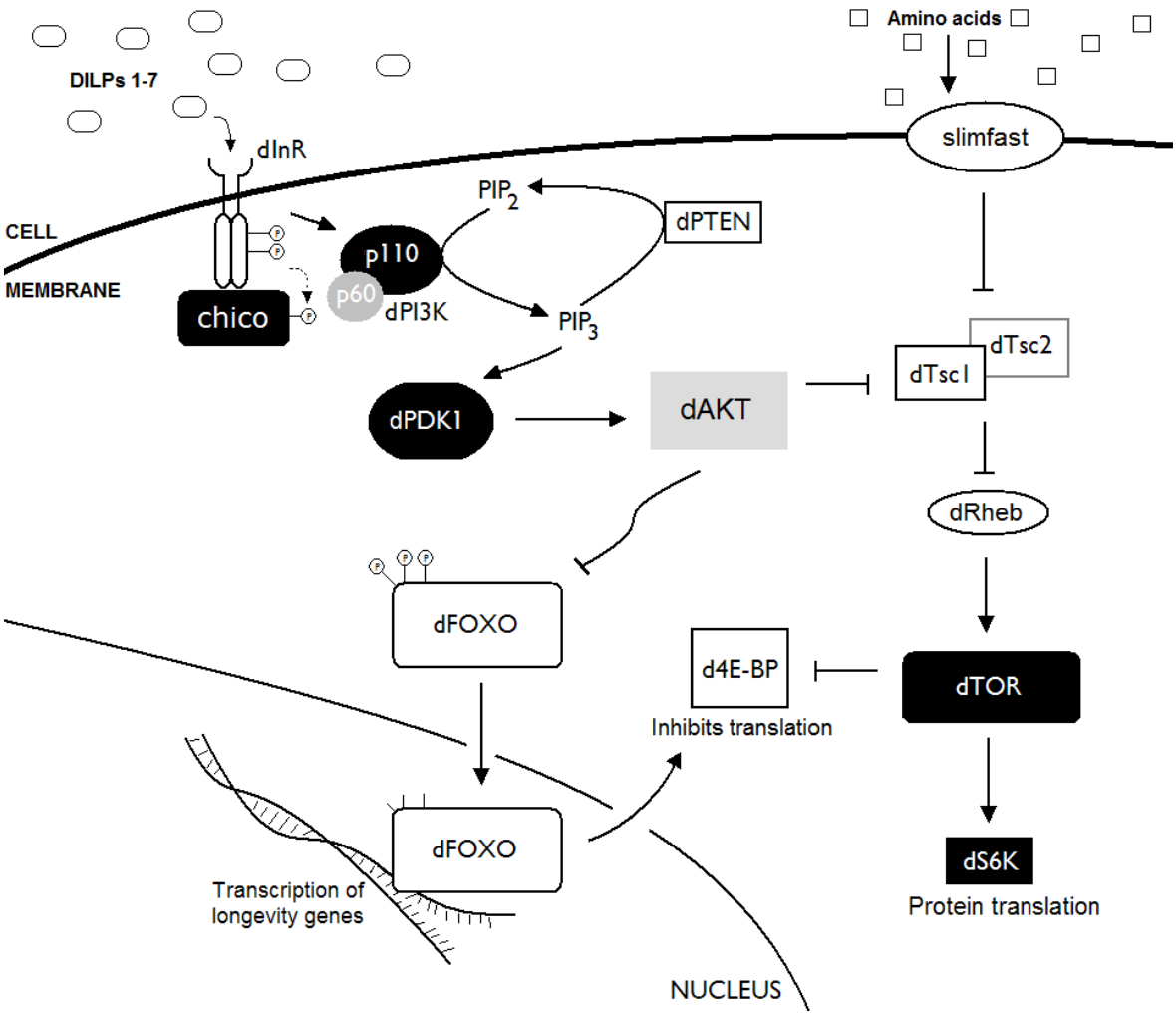

Figure 6.1.1 The *Drosophila* IIS pathway.
Shown are the known components of the *Drosophila* IIS pathway. *Drosophila* has single genes encoding all components of the IIS pathway apart from the *Drosophila* insulin-like peptides, for which seven genomic sequences have been identified (DILP1–DILP7). There is a single *Drosophila* insulin receptor, dINR, which transduces the signal from the DILPs to the lipid dPI3K, either directly or through the single *Drosophila* insulin receptor substrate, CHICO. Dp110 is the catalytic subunit and Dp60 is the regulatory subunit of dPI3K, which converts PIP_2 to PIP_3. The action of PI3K is antagonized by dPTEN, which degrades PIP2 to PIP_3. PIP_3 then activates a series of kinases, such as dPDK1 and dAKT, which subsequently phosphorylate the transcription factor dFOXO (Junger *et al*., 2003; Puig *et al*., 2003), leading to its inactivation and translocation to the cytoplasm. The TOR pathway controls amino acid sensing in the larval fat-body through the amino acid transporter slimfast (Colombani *et al*., 2003). The TOR kinase activates the downstream S6 kinase (which phosphorylates the small ribosomal subunit S6) to control growth in a nutrient-sensing pathway. The small GTPase dRheb (Ras homologue enriched in brain) is a positive effector of growth that has been shown by biochemical and genetic analysis to be downstream of dTSC1/2 in the TOR pathway (Stocker *et al*., 2003). Phosphorylation by TOR also inhibits the activity of 4EBP (eukaryotic initiation factor 4E-binding protein), which enables the mRNA cap-binding protein eIF4E (eukaryotic initiation factor 4E) to bind mRNAs, ultimately leading to increased initiation of translation. The IIS and TOR pathways interact at the level of dAKT, which phosphorylates TSC2, a negative regulator of TOR, in addition to dFOXO, thereby activating the TOR pathway.

The fact that homozygous null mutations in either *dPI3K* or *dAkt* cause lethality in development (Staveley *et al*., 1998; Weinkove *et al*., 1999) may be the reason why to date

no study has shown increased lifespan in flies with decreased dAkt signalling. Heterozygote null mutants with reduced activity in dPI3K or dAKT are not long-lived (Clancy *et al*., 2001), although head-fat-body-specific over-expression of *dPTEN*, an antagonist of *dPI3K*, results in a 20% increase in lifespan. However, in the same study, fat-body–specific over-expression of *dPTEN* in the abdomen and thorax did not extend lifespan (Hwangbo *et al*., 2004). This suggests reduction of either dPI3K or dAKT activity may still lead to dFOXO mediated lifespan extension in the fruit fly but it may be dependent upon the level of activity reduction and the specific tissue it is reduced in. Indeed, due to the pleiotropic nature of IIS, an intermediate level of IIS activity may maximise lifespan.

The role of Dp110/dAKT signalling in lifespan has not been as well documented as it has in terms of growth (Weinkove and Leevers, 2000). Only in recent years has the role of dFOXO in lifespan and stress resistance been discovered, and it is unclear to what degree the activity of dFOXO is regulated by dAkt. Even in other model organisms, the relationship between FOXO and Akt is unclear. For example, mammalian FOXO3a is not only regulated by Akt but also by a closely related serine/threonine kinase family known as serum and glucocorticoid-inducible kinases (SGKs). The SGK protein is dependent on PI3K activity (Park *et al*., 1999), and like Akt, is directly activated by PDK1 (Biondi *et al*., 2001). There are three main sites of phosphorylation on FOXO3a; and SGK is shown to phosphorylate FOXO3a at a different site to Akt. SGK favours the Ser-315 site, whereas Akt favours phosphorylation at the Ser-253 site. Thr-32, is phosphorylated by both Akt and SGK (Brunet *et al*., 2001). This suggests that SGK and Akt may cooperate or function in parallel to promote a variety of biological responses via FOXO3a. SGK-1 activity in the nematode is also shown to depend on PDK1 activation, and has a role in regulating DAF-16. Loss of *sgk-1* mutants have lower fecundity, increased stress resistance and extended lifespan, whereas *akt-1* and *akt-2* mutations result in the formation of dauers (Hertweck *et al*., 2004). Although, double gene knockdown by RNA interference (RNAi) against *akt-1* and *akt-2* in the sensitized *rrf-3* mutant background, which enhances the effects of RNAi, resulted in lifespan extension (Baumeister *et al*., 2006). The *Drosophila* homologue of SGK either does not exist or has yet to be identified.

In this chapter, I attempt to resolve whether dAkt plays a role in fly lifespan, fecundity or stress resistance, by identifying and studying mutants with varying degrees of activity in dAkt, and also in lines which have dAkt expression knocked-down by RNAi.

6.2. Methods

6.2.1. Fly stocks

The P[Switch] $S_1$106 driver (*w*; $S_1$106), which expresses Gal4 in the abdominal fat body and the gut (Poirier *et al*., 2008) via RU486 regulation (Osterwalder *et al*., 2001; Nicholson *et al*., 2008), and the ubiquitous GAL4 driver *daughterless*-GAL4 were originally obtained many years ago from the Bloomington Stock Centre. *GeneSwitch* Gal4 *w*; tubulin (*w*; *tubGS*) expresses RU486-regulated Gal4 under the control of the tubulin enhancer (S. Pletcher, gift from and personal communication). *yw; dAkt* ^{5F3}, *yw; dAkt* ^{6w2}, *yw; dAkt* ^{152} and *yw; dAkt*^{1} /TM3.Sb were a kind gift from the H.Stocker and E.Hafen lab (Stocker *et al*., 2002).

The RNA interference (RNAi) strain for *dAkt* (*w*;; dAkt-RNAi) and *dPI3K* (*w*;; T1 and *w,* T2) were obtained from the Vienna *Drosophila* RNAi Centre (2902, 38985 and 38986) (Dietzl *et al*., 2007). The *w*; T3 strain for *dPI3K* RNAi was a gift from H.Stocker. The RNAi constructs described contain the upstream activation sequence (UAS) necessary in the binary GAL4/UAS expression system (Brand and Perrimon, 1993) to activate gene knockdown.

6.2.2. Experimental food preparation

SYBrewer's food medium was prepared as described in chapter 2.

RU486 (mifepristone)

RU486 [Sigma-Aldrich, UK] was dissolved in 80% ethanol to make a 100mM stock solution. 1mL and 2mL volumes of 100mM RU486 stock solution was added to 1L of *Drosophila* food to make 100µM and 200µM (respectively) concentrations for

experiments. Food was stirred and dispensed 5mL per vial (35mL capacity) immediately after RU486 addition. Control food contained 2mL 80% ethanol per 1L of food.

Paraquat (methyl viologen dichloride)

5g paraquat [Sigma-Aldrich, UK] was dissolved in 1mL of distilled water and added to 1L of *Drosophila* food to make 20mM paraquat containing experimental food. 1.5mL of food was dispensed into vials and allowed to set.

6.2.3. Backcrossing flies

All experimental lines were backcrossed at least ten times into both w^{1118} and $w^{Dahomey}$ control backgrounds as described in **2.3.4**.

Harmful second-site mutations may exist in *dAkt* mutants as a result of the EMS mutagenesis used to create point mutations. In order to follow the mutation, flies were backcrossed against a P-element [15025] 1.5kb upstream of *dAkt* obtained from the Bloomington Stock Centre (**Figure 6.2.1**). This P-element was first backcrossed at least ten times into both w^{1118} and $w^{Dahomey}$ control backgrounds. The point mutations in backcrossed stocks were verified by sequencing (**6.2.9**.).

6.2.4. Stress tests

RNAi experiment and control females were collected two days after eclosion and transferred to 1x SY food medium containing the relevant RU486 concentration until day 7 of age, where they were tested for survival on (*i*) food medium containing 20mM paraquat (oxidative stress) (*ii*) 1% agar with addition of the appropriate concentration of RU486 (starvation).

dAkt and control males and females at day 7 of age were subjected to (*i*) food medium containing 20mM paraquat (oxidative stress) (*ii*) 1% agar only (starvation) (*iii*) normal food medium in a hyperbaric chamber with 90% oxygen (hyperoxia).

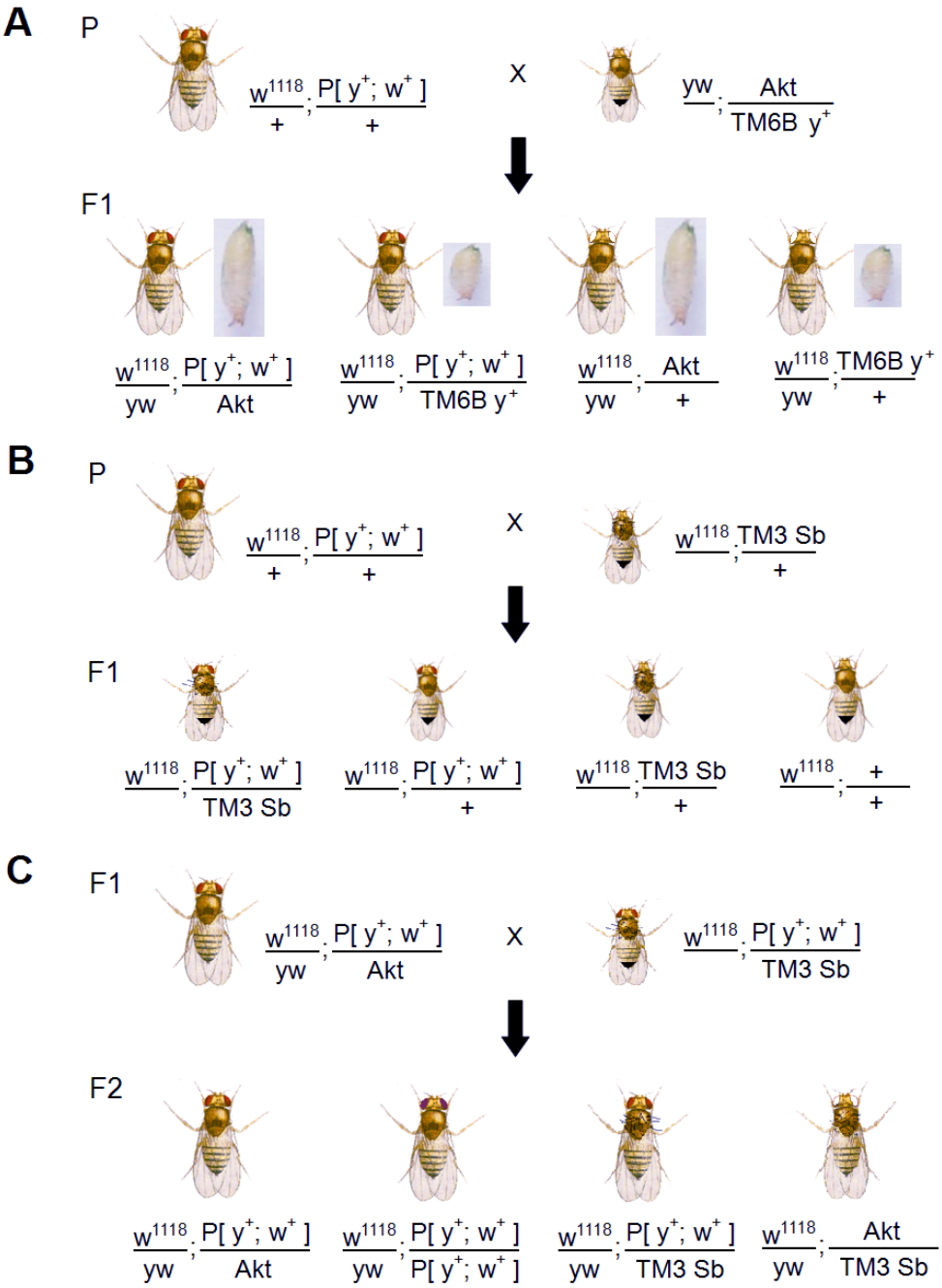

Figure 6.2.1 Backcrossing scheme for *dAkt* mutants.
This scheme relies on the close proximity of the P-element and the *dAkt* gene on the same chromosome, which reduces the chance of recombination occurring. Thus, the red-eyed phenotype of the P-element may be used as a marker to track the *dAkt* gene. Males are denoted by their smaller size and black tipped abdomens. (**A**) Virgin P-element females which have been previously backcrossed into the w^{1118} background for 10 generations are crossed with *dAkt* mutant males. In the F1 generation, virgin females which are red-eyed and pupated normally (first from left) are collected. (B) Virgin P-element females are crossed with balancer TM3 males with stubble phenotype. Both lines have been previously backcrossed into the w^{1118} background for 10 generations. In the F1 generation, red-eyed males with stubble phenotype (first from left) are collected. (**C**) The females and males collected from crosses **A** and **B** were mated to produce the F2 generation, where red-eyed, normal-bristled virgin females (first from left) were collected. Double P-element females can be distinguished by their darker red eye pigmentation (second from left). Collected virgin females were crossed with males collected from cross **B**, i.e. cross **C** was repeated for a further 10 generations.

6.2.5. Metabolic measurements

Trehalose

Flies at day 7 of age were homogenised in 0.2 M sodium carbonate and heated at 95^{o}c for 2 hours. The pH was adjusted to 5.2 by addition of acetic acid and sodium acetate, followed by addition of trehalase (0.05 units/ml)[Sigma-Aldrich, UK] and left at 37 ^{o}c overnight. The liberated glucose amount was determined by adding Infinity Glucose Reagent (ThermoElectron) and the absorbance of the liquid sample was then measured at 340nm [Hitachi U-2001 Spectrophotometer (Lambda Advanced Technology Ltd., UK)].

Glycogen

Flies at day 7 of age were homogenised in saturated sodium sulphate solution, centrifuged for 1min at 10,000rpm, and the supernatant transferred to chloroform: methanol (1: 1) solution. The solution was inverted several times and centrifuged for 5mins at 10,000 rpm. The supernatant was discarded and the pellet was incubated for 20 minutes at 90 ^{o}c in 1ml of anthrone solution (71mg in 50mL 70% sulphuric acid). The level of glycogen was determined by measuring the absorbance of the liquid sample at 340nm [Hitachi U-2001 Spectrophotometer (Lambda Advanced Technology Ltd., UK)].

Triglycerides

Flies at day 7 of age were homogenised in 0.05% Tween and centrifuged for 3min at 14,000rpm. The supernatant was collected and the level of lipid was determined by adding Infinity Triglyceride Reagent (ThermoElectron) and the absorbance of the liquid sample was then measured at 540nm [Hitachi U-2001 Spectrophotometer (Lambda Advanced Technology Ltd., UK)].

6.2.6. Quantitative reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted from snap-frozen the whole body of 7-day old males by using TRIzol (GIBCO) as described in section 2.4. mRNA in total RNA was reverse transcribed by using oligo(dT) primer and the Superscript II system (Invitrogen). RT-PCR was performed with the PRISM7000 sequence-detection system with *POWER*® SYBR Green PCR Master

Mix (Applied Biosystems, Foster City, CA, USA) by following the maunfacturer's instructions.

The *dAkt* primers were:

dAkt _for: CCG TTC CTC ATT TCA CTC AA

dAkt _rev: GAT AGC CCA GAG CAG AAA TG

Endogenous control primers were:

*actin5c*_for: CAC ACC AAA TCT TAC AAA ATG TGT

*actin5c*_rev: AAT CCG GCC TTG CAC ATG

Primers were optimised following ABI procedures and relative quantities of *dAkt* transcripts were determined using the relative standard curve method and normalisation to *actin5c*. Fold change calculations and absolute quantification for *dAkt* expression was followed as described in (Broughton *et al*., 2005).

6.2.7. Immunoprecipitation and assay of dAKT activity

The following procedure was adapted from (Alessi *et al*., 1996; Staveley *et al*., 1998) (**Figure 6.2.2**). Females from the w^{1118} background were homogenised in lysis buffer (50mM Tris/HCL pH7.5, 1mM EDTA, 1% Triton X-100, 0.27M sucrose, 5mM ß-glycerolphosphate, 1mM sodium orthovanadate, 50mM sodium fluoride, 0.1% ß-mercaptoethanol, 1:200 Complete™ Protease Inhibitor) and centrifuged for 15mins, 14,000rpm and 4^{o}c. The supernatant was collected and rotated for 1 hour at 4^{o}c with protein G-sepharose beads [Sigma] as a pre-clearing step; the level of protein in the supernatant was then determined by the Bradford assay. 500µg of protein extract was then rotated for 2 hours at 4^{o}c with protein G-sepharose beads, which were coupled to 10µg of anti-Akt antibody [9272, Cell Signaling Technology]. Beads were washed twice with lysis buffer containing 0.5M NaCl, and then twice with Buffer A (50mM Tris/HCL pH7.5, 0.1mM EGTA, 0.1% ß-mercaptoethanol).

Beads conjugated with dAKT protein were incubated at 30^{o}c for 30 minutes in 30µL buffer [50mM Tris/HCL pH7.5, 10mM magnesium acetate, 0.1mM EGTA, 5mM ß-glycerolphosphate, 1mM sodium orthovanadate, 0.1% ß-mercaptoethanol, 2.5µM protein kinase inhibitor, 250µM ATP, 100µM Crosstide (GRPRTSSFAEG) and 10µCi total [γ-^{32}P] ATP (Perkin-Elmer, 3000Ci(111Tbq)/mmol, 10mCi/mL)]. During this period, the Akt protein

phosphorylated Crosstide with γ-^{32}P. Phosphocellulose paper (P81 Whatman paper, VWR) was immersed in 1% phosphoric acid and dried. 10µL of the buffer containing γ-^{32}P - Crosstide was transferred onto the Whatman paper, dried and then washed 2x 5 minutes and 2x 15 minutes in 1% phosphoric acid. The paper was transferred to 1mL of scintillant and analysed for ^{32}P radioactivity. Dynal® magnets (Invitrogen) were used at all times to isolate and separate the G-sepharose beads from the liquid mixture.

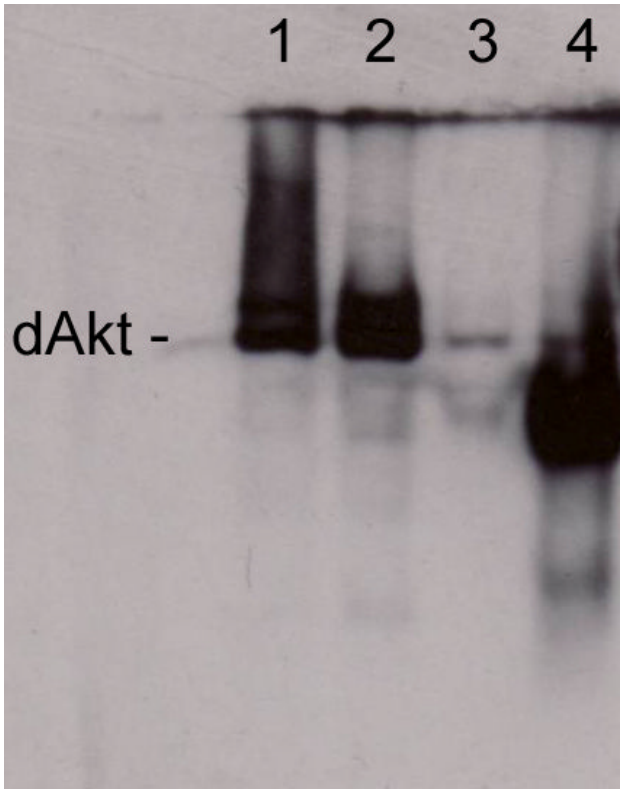

Figure 6.2.2 SDS-PAGE and western blotting of protein extracts during various stages of the immunoprecipitation.
Protein was immunoblotted with anti-AKT antibody (9272, Cell Signaling Technology). Lane 1 displays 500µg of protein in fly homogenate before the pre-clearing step. Lane 2 displays 500µg of protein in the homogenate after pre-clearing with protein G-sepharose beads. Lane 3 displays 500µg of protein in the homogenate after immunoprecipitation with protein G-sepharose beads conjugated with AKT antibody. Some AKT protein still remains. Lane 4 displays the dAKT protein that was conjugated out of the homogenate mixture by immunoprecipitation. The protein was obtained by placing the protein G-sepharose beads conjugated with dAKT protein in 30µL of loading buffer at 95^{o}C for 5 minutes. The large smear may be a result of this stripping process. Blots indicate that a large amount of dAKT protein was successfully extracted from the homogenate by immunoprecipitation. A band of dAKT protein is visible within the smear in lane 4.

6.2.8. Immunoblotting

Western blots were performed to measure phosphorylated and unphosphorylated levels of dAkt from homogenate extracts during the immunoprecipitation and dAkt activity assays. For protein level detection, whole fly extracts were obtained from experiment and control flies (w^{1118} females) by ribolysing 20 flies in 200µL of 20% trichloroacetic acid, removing sample solution and centrifuging for 15 minutes at 14000RPM at 4^{o}c. The white protein pellet was resuspended in a loading buffer (150mM Tris pH9.0, 4% SDS, 30% glycerol, 100mM DTT and 0.01% bromophenol blue). 1M Tris was added until the sample turned blue if yellow. The sample was heated at 80^{o}c for 15

minutes and centrifuged for 15 minutes at 14000RPM at room temperature. The supernatant was collected and the protein was quantified using the Bradford protein assay (Bio-Rad protein assay reagent; Bio-Rad Laboratories (UK) Ltd, Hemel Hempstead) according to the manufacturer's instructions (see **2.4**). Equal amounts of protein were loaded for each sample for 8% SDS-polyacrylamide gel electrophoresis (PAGE) with Rainbow™ molecular weight marker (Amersham plc, Little Chalfont, UK). The gels were blotted according to section 2.4 and incubated with either anti-dFOXO antibody at (Giannakou *et al*., 2007), anti-phospho-GSK-3α/β [9331, Cell Signaling Technology, NEB, UK], anti-Akt antibody [9272, Cell Signaling Technology, NEB, UK] or anti-tubulin antibody [rat monoclonal (YL1/2) to tubulin, Abcam, Cambridge, UK]. Anti-horseradish peroxidase (HRP) secondary antibodies were used and the signals detected by chemiluminescence using ECL kit (Amersham plc). Densitometry of blots were carried out in ImageJ (National Institutes of Health, USA) (Gassmann *et al*., 2009).

6.2.9. Sequencing

Flies (*N* = 20) were sampled and genomic DNA was extracted (**section 2.4**), but adapted using QIAGEN Qiawell and QIAprep. The *dAkt* gene was amplified and isolated by PCR into 1kb overlapping fragments. These template fragments were purified by QIAGEN Qiaquick PCR protocol into 10µL volumes (10ng/µL) per sequencing reaction. Primers were designed for these 1kb templates, whereby 500bp fragments could be produced during the sequencing run. Sequencing reactions were performed by the Wolfson Institute for Biomedical Research, UCL. Data analyses were performed using Lasergene Seqman Pro (DNASTAR) software. Single nucleotide exchanges were verified by PCR.

6.3. Results: *dAkt* mutants

6.3.1. Reduced catalytic activity in *dAkt* mutants

Akt (also known as protein kinase B, PKB) is an important serine/threonine kinase in insulin/ insulin-like growth factor-1 signalling (IIS). AKT is bound to plasma membrane via its PH domain by PIP_3, a product of PI3K activity. At the plasma membrane, AKT is

activated upon phosphorylation by PDK1 (Rintelen *et al*., 2001). In *Drosophila*, a single point mutation in the PH domain can lead to a 30% decrease in dAKT activity in mutant larvae, $dAkt^{3}$ (Stocker *et al*., 2002), whereas null mutants, $dAkt^{1}$, produce catalytically inactive proteins and are non-viable (Staveley *et al*., 1998; Stocker *et al*., 2002). Thus, in order to measure the effects of reduction of *dAkt* activity on lifespan hypomorphic mutants are needed.

Three homozygous viable hypomorphic mutations $dAkt^{5F3}$, $dAkt^{6w2}$, $dAkt^{152}$ were generated by EMS mutagenesis and selected through growth screens in the Hafen lab (E.Hafen, personal communication). Characterisation of the mutants begun by sequencing genomic DNA for the *dAkt* gene extracted from the hypomorphs, which revealed single nucleotide exchanges resulting in single amino acid substitutions in either the PH or kinase domain (**Figure 6.3.1A**). For $dAkt^{5F3}$, an evolutionary conserved alanine residue was replaced by a non-conserved valine (A295V) in the first alpha helix of the kinase domain. For $dAkt^{6w2}$ and $dAkt^{152}$, a substitution of arginine with a histine (R43H) in the third beta-sheet and a glycine with a glutamic acid (G30E) in the second beta-sheet of the PH domain was observed respectively. To address how these mutations affected *dAkt* activity, the *dAkt* alleles were combined with $dAkt^{1}$ producing viable adult dwarf flies, and the activity of the kinase extracted from these flies was compared to that in the wild-type. Akt activity in heterozygotes of $dAkt^{1}$ was also measured. The level of dAkt protein did not differ between the mutant genotypes (**Figure 6.3.1B**) but activity was significantly reduced compared to the $dAkt^{1}$ heterozygote and the wild-type ($P < 0.005$ and $P < 0.0001$, respectively, Wilcoxon). The activity did not differ significantly among the *dAkt* allelic null mutants, and $dAkt^{1}$ heterozygotes had reduced *dAkt* activity compared to w^{1118} wild-type flies ($P < 0.05$, Wilcoxon).

The effect of dAkt reduction on downstream signalling in mutants was analysed by SDS-PAGE and western blot of whole fly protein extracts **(Figure 6.3.1C-D**). Compared to the w^{1118} wild-type, levels of phosphorylated and unphosphorylated dFOXO were the least in $dAkt^{6W2/-}$ and $dAkt^{152/-}$, marginally lower in $dAkt^{5F3/-}$, and unchanged in $dAkt^{+/-}$. Levels of phosphorylated SHAGGY *(*the *Drosophila* GSK-3 homologue) were also mildly reduced in all mutants compared to the wild-type (Cross *et al*., 1995). Despite insignificant difference in dAKT activity between the mutants, the strongest reduction in dAKT activity, $dAkt^{5F3/-}$, did not have a great effect on dFOXO phosphorylation but did on

SHAGGY phosphorylation. However, the size and weight, in the next section, implies that *dAkt*$^{5F3/-}$ had the weakest effect compared to *dAkt*$^{6W2/-}$ and *dAkt*$^{152/-}$. This suggests that the reduction of dAKT activity may trigger a negative feedback response either via the activation of dTSC (Potter *et al*., 2002) or through an unknown pathway. It has been observed in larvae that loss of dTSC function results in dAKT inhibition via dS6K (Radimerski *et al*., 2002a), although it has also been shown that dS6K activation does not require dAKT inhibition (Radimerski *et al*., 2002b).

6.3.2. Reduced dAKT activity and morphology/physiology

The insulin signalling pathway is known to regulate cellular metabolism and energy homeostasis in vertebrates, as well as size and growth in *Drosophila* (Bohni *et al*., 1999; Goberdhan *et al*., 1999). Consistent with these findings, reduced body size and mass was observed in the hypomorphic mutants of *dAkt* in three different backgrounds (**Table 6.1** and **Figure 6.3.1E-F**). Compared to wild-type controls of the same age, *dAkt*$^{5F3/-}$ had a 41% weight reduction in females and 46% in males; *dAkt*$^{6w2/-}$ were reduced by 61% in both males and females; *dAkt*$^{152/-}$ were reduced by 64% in females and 58% in males. *dAkt*^{1} heterozygotes were reduced by 9% and 12% in females, 21% and 5% ($P < 0.005$, Wilcoxon) in males in the *yw* and *w*^{1118} backgrounds, respectively. There was no significant difference between *dAkt*^{1} heterozygotes and wild-type controls in *w*^{Dahomey} (**Table 6.1** and **Figure 6.3.1F**).

A linear regression model was fitted by least squares to dAKT catalytic activity in the hypomorphic *dAkt* mutants in both backgrounds. This was then used to predict the effect altering kinase activity had on mass (**Figure 6.3.2A-B)**, the level of phosphorylation in downstream targets (*w*^{1118} background only, **Figure 6.3.2C-D**). Linear regression analysis indicated that there was a positive relationship between dAKT activity and mass in both sexes from both backgrounds. Interestingly, no significant relationship between dAKT activity and FOXO phosphorylation was found in the *w*^{1118} background, which suggests that the growth reduction observed, may be because either reduced dAKT activity is correlated with decreased SHAGGY phosphorylation or altered growth via another

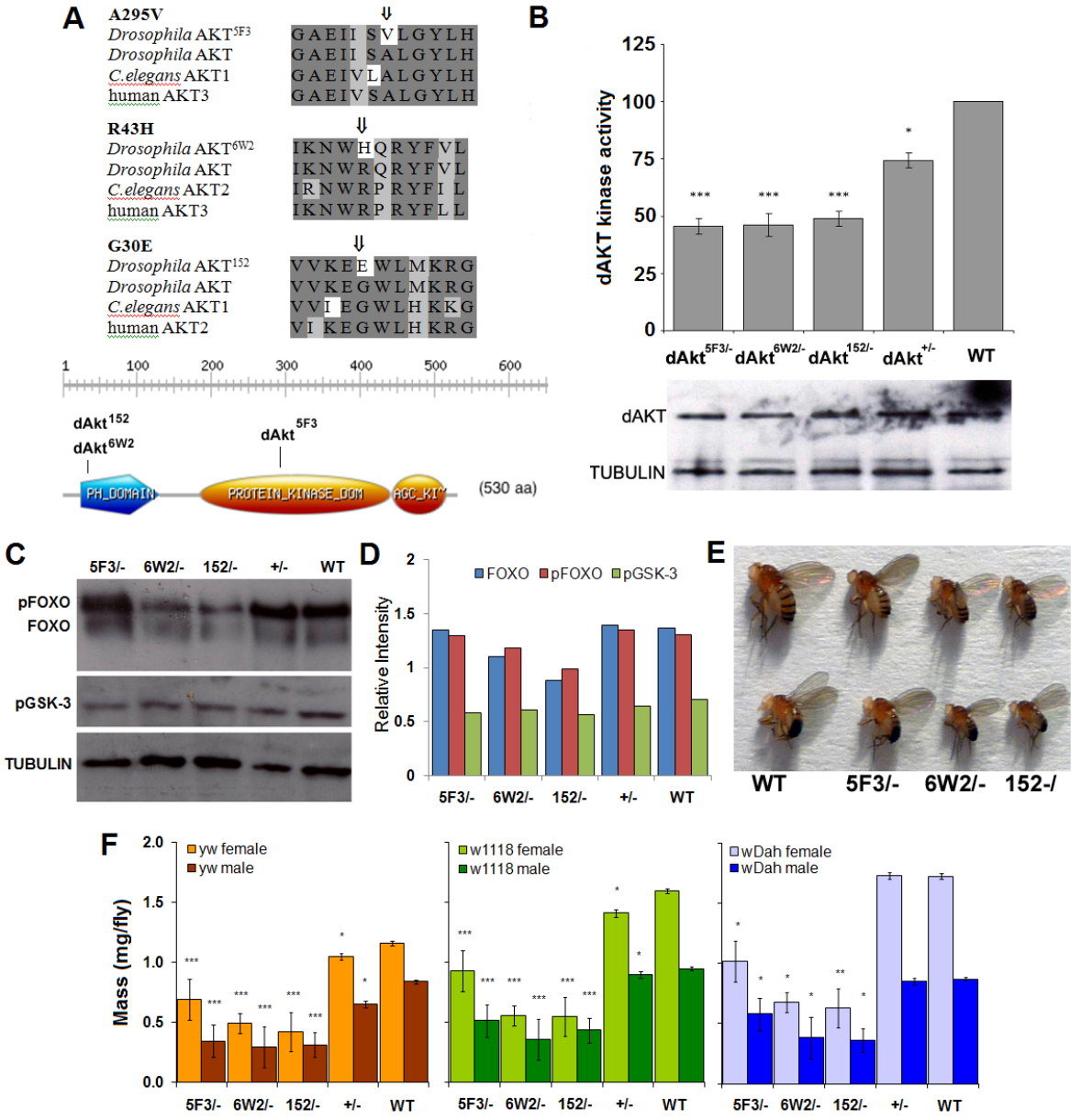

Figure 6.3.1 The effect of reduced dAKT activity.
In all panels, significant differences compared to wild-type (WT) are indicated as $P < 0.05$ (*), $P < 0.001$ (**), $P < 0.0001$ (***). Bars: mean ± SEM. (**A**) A comparison between the amino acid sequences of dAKT mutants and the WT form in three different species. Arrows indicate the amino acid substitutions in mutants. Dark grey residues are conserved; light grey residues share similar properties and white residues have different properties. The substitutions *in situ* to the protein are also displayed. (**B**) The effect of the amino acid substitution on activity in dAKT extracted from adults of crossings between allele mutants and the null mutant, $dAkt^{1}$. Kinase activity from null heterozygotes was also measured. Activity from w^{1118} WT adult was considered to be 100%. dAkt protein was detected in 500µg of fly extract using the same antibody used in the kinase assay, total AKT. Tubulin protein was also detected as a loading control. (**C**) From top to bottom: protein levels of *dFOXO*, *shaggy* and *tubulin* in w^{1118} females of (from left to right) $dAkt^{5F3/-}$, $dAkt^{6W2/-}$, $dAkt^{152/-}dAkt^{1}$ heterozygotes and WT. Whole fly protein extracts were subjected to SDS-PAGE and immunoblotted with dFOXO, phospho-GSK-3β and tubulin antibodies. (**D**) The relative intensity of the downstream components of dAKT compared to tubulin standard in the western. (**E**) Flies are, from left to right: w^{1118} (WT), $dAkt^{5F3/-}$, $dAkt^{6W2/-}$ and $dAkt^{152/-}$. Females are top and males are bottom. Flies were obtained by crossing allelic *dAkt/TM3.Sb* with $dAkt^{1}$/*TM3.Sb* (null over balancer). (**F**) The mean mass of individual flies ($N = 100$) was calculated for both sexes in three different backgrounds: *yw*, w^{1118}, $w^{Dahomey}$.

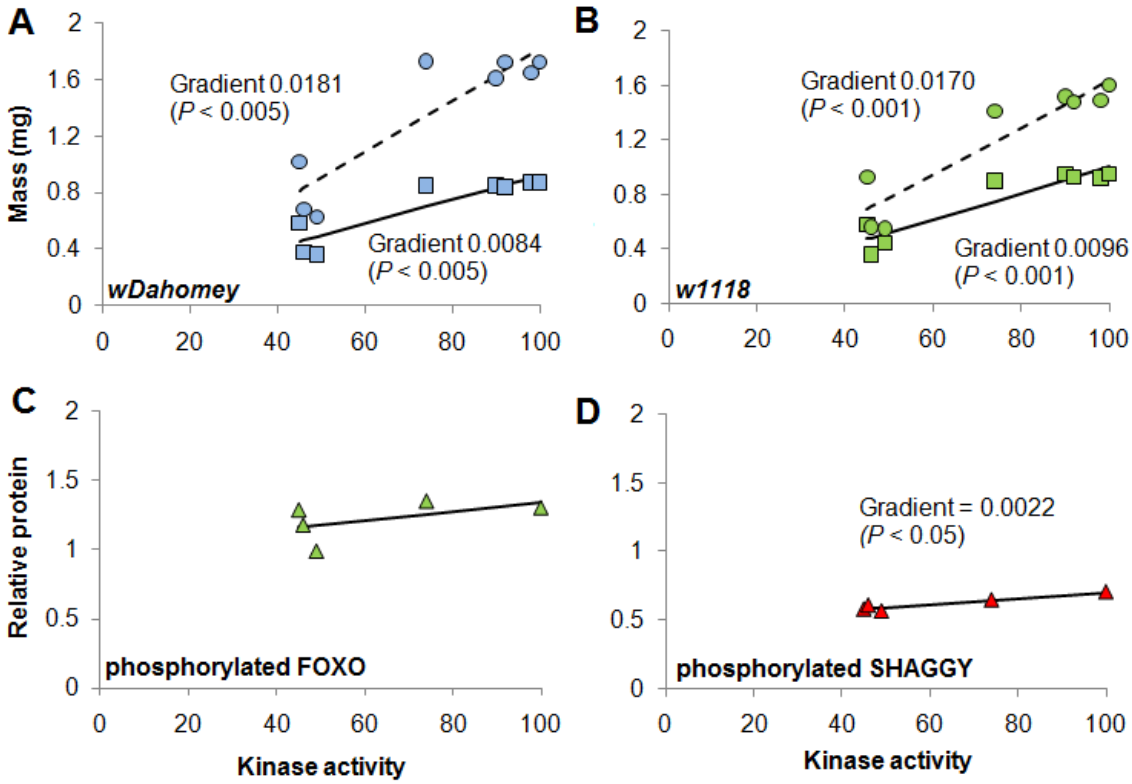

Figure 6.3.2 The relationship of dAKT kinase activity with growth and downstream phosphorylation targets.

The kinase activity of each genotype in each background and sex was analysed by linear regression**.** A significant positive relationship between the level of dAKT activity and growth size was found in both sexes in the (**A**) $w^{Dahomey}$ and (**B**) w^{1118} backgrounds. Points represent observed male (square) and female (circle) values, whereas lines represent the predicted slope and intercept from the analysis. The amount of downstream phosphorylation was calculated from Western blots relative to tubulin levels (**C**) No significant relationship was found between kinase activity and phosphorylated FOXO in w^{1118}. (**D**) A significant positive relationship was found between kinase activity and phosphorylated SHAGGY in w^{1118}.

pathway such as S6K. SHAGGY is known to inhibit glycogen synthase (Cohen and Frame, 2001), thus its reduction may also explain the increased levels of glycogen molecules found in *dAkt* mutants.

Additionally, reports have suggested that disruption to IIS in *Drosophila* leads to increased levels of the three major energy storage molecules: triglycerides (Bohni *et al*., 1999; Tatar *et al*., 2001), glycogen and trehalose (Broughton *et al*., 2005). The levels of triglyceride, glycogen and trehalose per unit of fresh weight were determined using whole-body extracts of flies. The triglyceride and trehalose levels in whole fly extracts were found to be significantly increased in both males and females compared to their controls (**Figure 6.3.3A-B**), while mild increases in glycogen content were also observed in experimental males and females (**Figure 6.3.3C**).

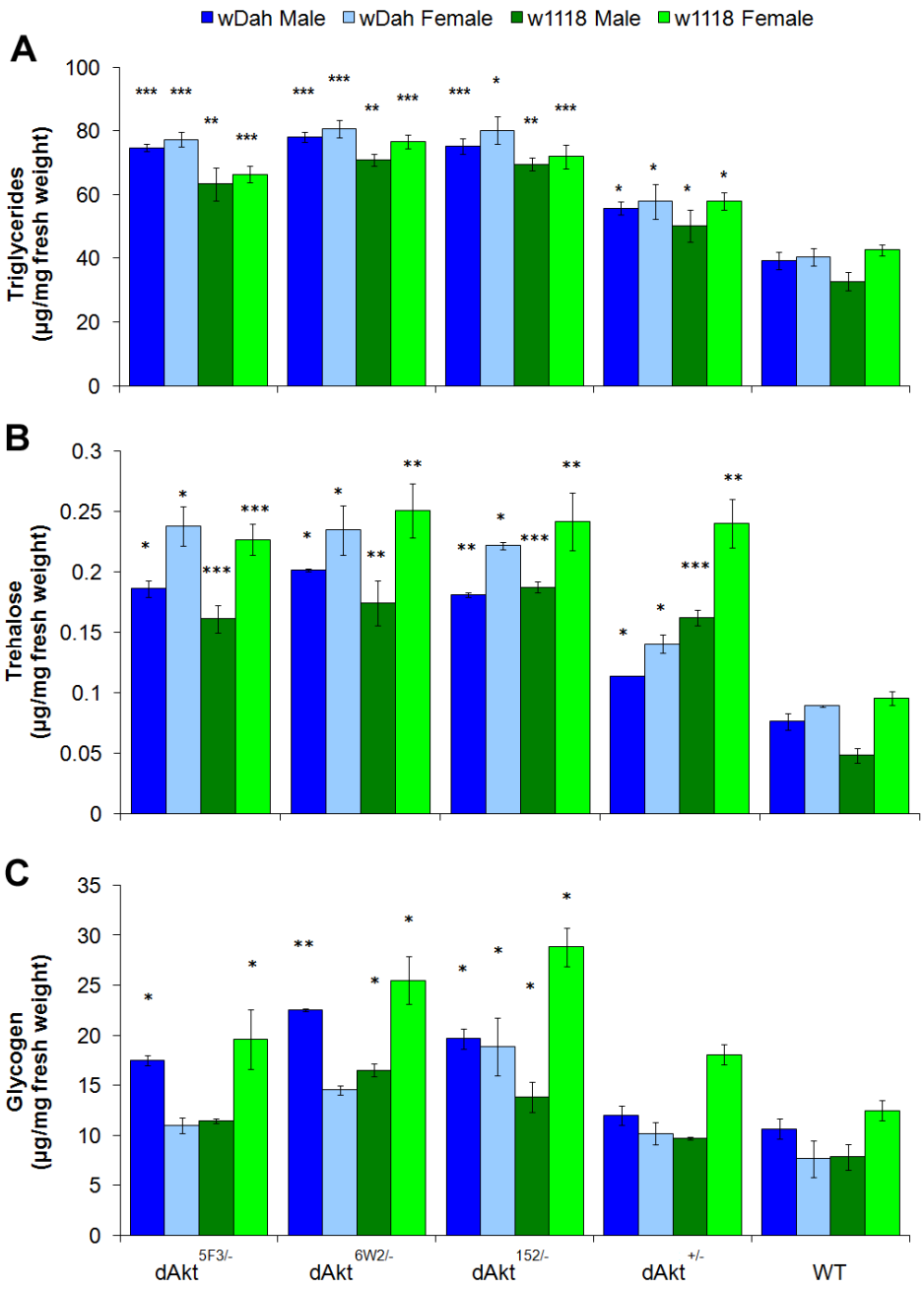

Figure 6.3.3 Mutant *dAkt* animals have increased levels of triglycerides, trehalose and glycogen.
Bars: mean ± SEM. In all panels, * = $P < 0.05$, ** = $P < 0.001$, *** = $P < 0.0001$ indicates significant difference compared to the wild-type (WT). (A) Triglyceride levels in *dAkt* mutants. $N = 30$ for each genotype. (B) Trehalose levels in *dAkt* mutants (C) Glycogen levels in *dAkt* mutants $N = 20$ for each genotype.

6.3.3. Reduced dAKT activity and lifespan/fecundity

A number of studies have shown that IIS reduction extends adult *Drosophila* lifespan (Clancy *et al.*, 2001; Tatar *et al.*, 2001; Broughton *et al.*, 2005). However, the effect on lifespan may depend on the degree to which signalling is reduced. It has been reported that severe disruption of IIS leads to an increase in premature mortality in adult *C.*

elegans but invariably leads to an increase in maximum lifespan (Guarente and Kenyon, 2000), and in *Drosophila*, the strong reduction of IIS in Inr^{GC25}/Inr^{E19} leads to a possible increase in age-independent mortality despite a reduction in age-specific mortality rate acceleration (Clancy *et al*., 2001). These early deaths indicate flies were sick and may mask the benefits of slowed ageing. This may explain why adult lifespan extension during *dAkt* disruption has never been recorded and why $dAkt^{3}$ homozygous adults, which have 30% dAkt activity of wild-type levels, are overall short-lived (Clancy *et al*., 2001; Stocker *et al*., 2002). To investigate these effects, the longevities of both male and mated female *dAkt* allelic mutants were assessed for survival (**Table 6.1**) and age-specific mortality (**Table 6.2**) in w^{Dahomey} (**Figure 6.3.4**) and w^{1118} (**Figure 6.3.5**) backgrounds.

$w^{Dahomey}$ background

$dAkt^{5F3/-}$ and $dAkt^{5F3/+}$ mutants exhibited mildly extended (+3 and +7%, respectively) but significant survival in males. Extended survival in males may be a result of decreased initial rate of mortality (α) because no change was detected in the slope of the mortality trajectory (β). Although, $dAkt^{5F3/-}$ females had a significantly decreased slope of the mortality trajectory, they also had a significantly increased initial rate of mortality, which may mask any extension of median lifespan. Indeed, $dAkt^{5F3/-}$ and $dAkt^{5F3/+}$ females were longer lived (+8% and +14%, respectively) than the wild-type, but these increases were not significant. The data suggest that for a "weaker" mutation (in terms of its effect on dFOXO and size) to extend lifespan in flies, it must target flies which are either more susceptible or more responsive to IIS disruption. In this case, males were found to be more responsive to $dAkt^{5F3}$ manipulation.

For a "stronger" mutation, $dAkt^{6W2/-}$, significant lifespan extension to males and females was both observed. $dAkt^{6W2/-}$ males had a 12% increase in lifespan and, like $dAkt^{5F3/-}$ males, were found to have a decreased initial rate of mortality but an increased slope in mortality trajectory. Although no differences in mortality were detected for $dAkt^{6W2/-}$ females, they had a 35% extension in lifespan.

Furthermore, in another "stronger" mutation, $dAkt^{152/-}$, females were also found to be 27% longer lived. In contrast, males did not exhibit any significant differences in lifespan, but were marginally shorter lived. Mortality analysis of $dAkt^{152/-}$ lifespan data revealed no significant differences when to the wild-type.

Finally, *dAkt*^{1} heterozygotes, which had the greatest level of dAKT activity of the flies crossed with a null allele, were found to have very mild but significantly extended lifespan in males (+3%) and females (+6%).

Overall, the data suggests disruption of dAKT signalling does extend lifespan. Females had larger increases of lifespan than males to *dAkt* mutation, but males were more consistent in their response of lifespan extension. This was reflected by the "strongest" *dAkt* mutations providing lifespan extension in both sexes, but more robustly in females, and the "weaker" mutations extending lifespan more often in males than females. As indicated by the mild increases of lifespan in male heterozygotes of each hypomorphic mutation. This data, however, is only applicable to flies in the $w^{Dahomey}$ background, thus it would be of great interest to see if this can be replicated in flies from the w^{1118} background.

w^{1118} background

Although, *dAkt*$^{5F3/-}$ females were significantly shorter lived, *dAkt*$^{5F3/-}$ and *dAkt*$^{5F3/+}$ males were observed with 10 and 13% (respectively) increases lifespan compared to the wild-type. Furthermore, *dAkt*$^{5F3/+}$ females were also observed with a 6% increase in lifespan. *dAkt*$^{6W2/-}$ females were found to have mild (+ 3%) but significantly increased lifespan. No significant differences in lifespan were found in any other mutation or sex in this background. This was reflected in the mortality analysis where no differences were observed in any flies with one exception, *dAkt*$^{6W2/-}$ females, which had an increased initial rate of mortality but a lowered slope in the mortality trajectory.

In summary, male *dAkt* mutants exhibited mild but significant lifespan extension in all cases in the $w^{Dahomey}$ background, except *dAkt*$^{152/-}$, where they were shorter lived than controls. Female *dAkt* mutants also exhibited mild lifespan extension in the majority of cases in the $w^{Dahomey}$ background, whereas in the w^{1118} background, the results were more variable. This suggests that the w^{1118} background are either more resistant to IIS disruption (i.e. not as responsive in terms of survival) or cannot tolerate IIS disruption (i.e. flies are more readily sick and thus masks any survival benefits). In the $w^{Dahomey}$ background, female hypomorphs over nulls were exhibiting lifespan extension of up to

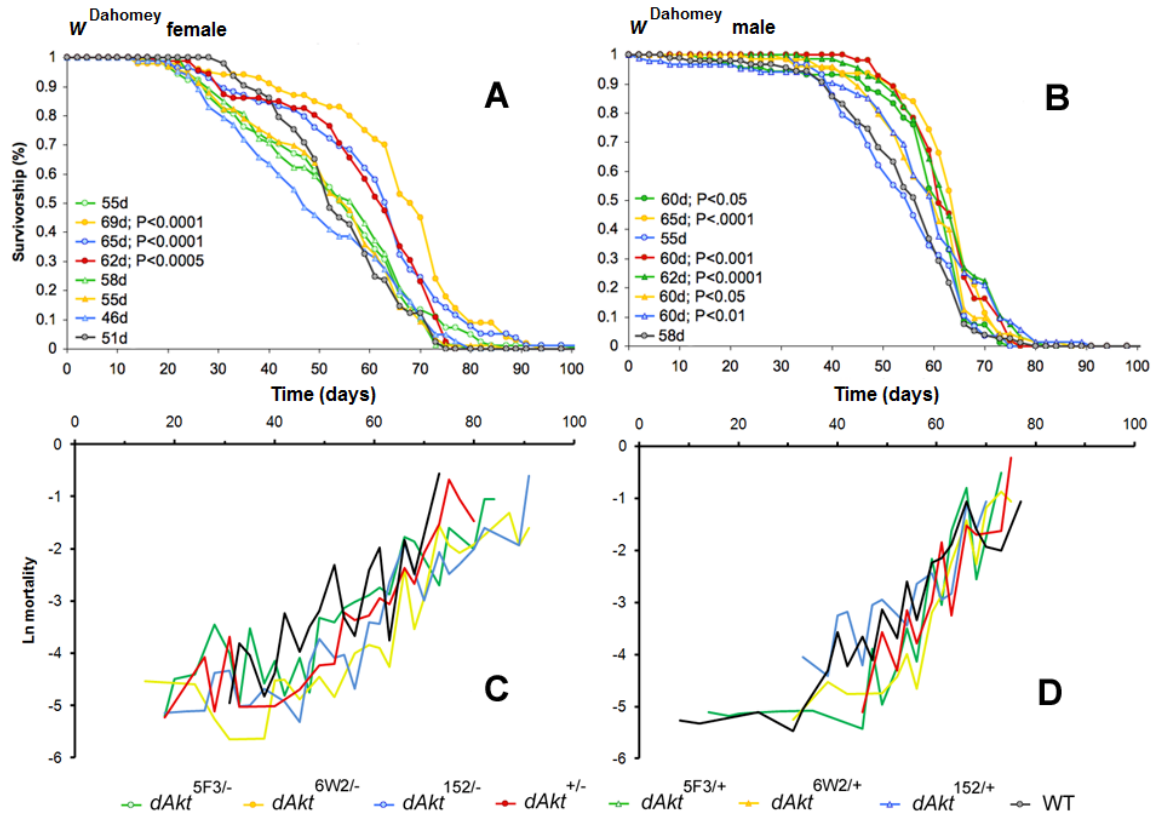

Figure 6.3.4 The effect of mutations of *dAkt* on female and male lifespan and fecundity in the $w^{Dahomey}$ background.
Each genotype was raised in parallel under the same conditions and assayed on 1x SY for lifespan. The graph legend reports the median lifespan in days and; *P*-value, if significant difference was observed from the log-rank test when compared to the WT. (**A**) Female lifespan in $w^{Dahomey}$ (**B**) Male lifespan in $w^{Dahomey}$ (**C**) Female mortality in $w^{Dahomey}$ (**D**) Male mortality in $w^{Dahomey}$. Data shown are from a single trial in which all lifespan experiments were run simultaneously.

	AKT	Mass (mg)				Median lifespan (days)			
	activity to	w^{1118}		$w^{Dahomey}$		w^{1118}		$w^{Dahomey}$	
Genotype	WT (%)	M	F	M	F	M	F	M	F
$dAkt^{5F3/-}$	**45**	**0.52**	**0.93**	**0.58**	**1.02**	**66**	**58**	**60**	55
$dAkt^{6W2/-}$	**46**	**0.36**	**0.56**	**0.38**	**0.68**	60	**62**	**65**	**69**
$dAkt^{152/-}$	**49**	**0.44**	**0.55**	**0.36**	**0.63**	62	56	55	**65**
$dAkt^{+/-}$	**74**	**0.90**	**1.41**	0.85	1.73	60	58	**60**	**62**
$dAkt^{5F3/+}$	-	0.95	1.52	0.85	1.61	**68**	**64**	**62**	58
$dAkt^{6W2/+}$	-	0.93	1.48	0.84	1.72	64	62	**60**	55
$dAkt^{152/+}$	-	0.92	1.49	0.87	1.65	58	**56**	**60**	46
Wild-type	100	0.95	1.60	0.87	1.72	60	60	58	51

Table 6.1 The dAKT kinase activity, mass and lifespan of three adult *dAkt* mutants.
Bold font denotes a significant difference ($P < 0.05$) compared to the wild-type. (M) male, (F) female.

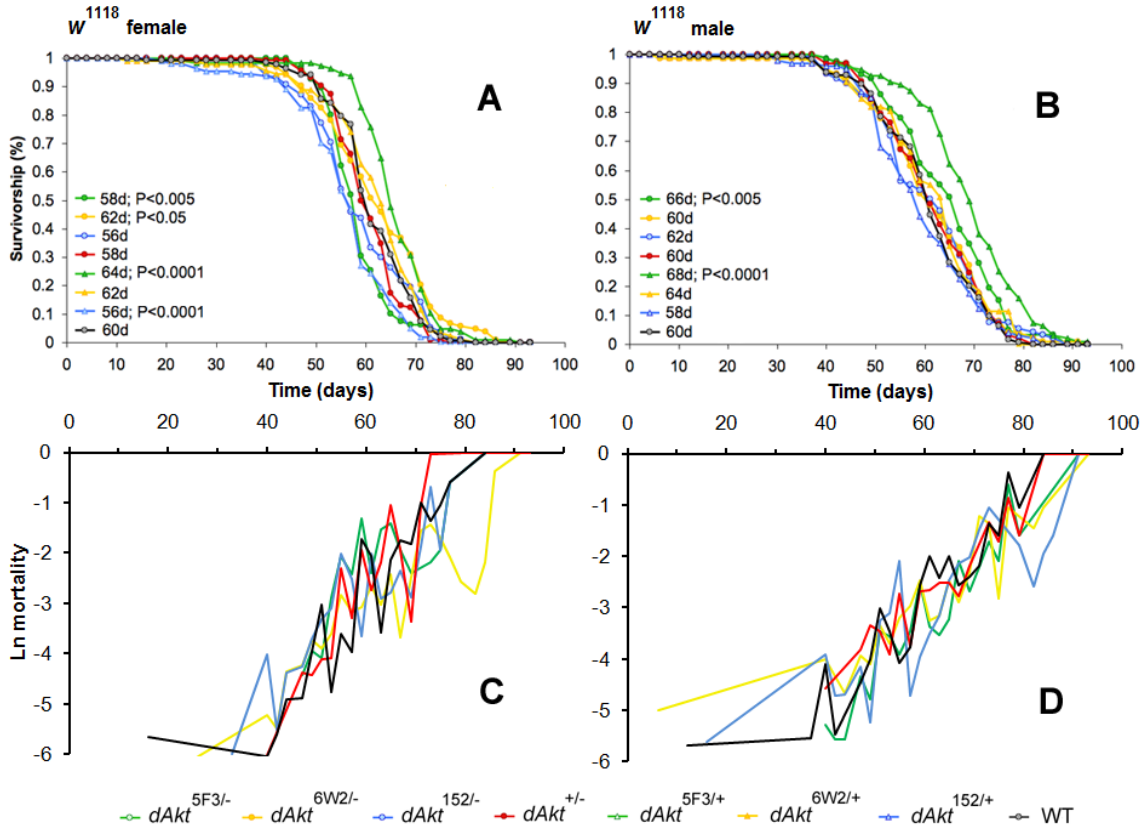

Figure 6.3.5 The effect of mutations of *dAkt* on female and male lifespan and fecundity in the w^{1118} background.
Each genotype was raised in parallel under the same conditions and assayed on 1x SY for lifespan. The graph legend reports the median lifespan in days and; *P*-value, if significant difference was observed from the log-rank test when compared to the WT. (**A**) Female lifespan in w^{1118} (**B**) Male lifespan in w^{1118} (**C**) Female mortality in w^{1118} (**D**) Male mortality in w^{1118}. Data shown are from a single trial in which all lifespan experiments were run simultaneously.

+35% ($dAkt^{6W2/-}$), whereas in males the maximum extension observed was only +12% ($dAkt^{6W2/-}$). Thus, males were less tolerant to IIS reduction than females as male lifespan did not respond as greatly as females to *dAkt* manipulation, although there was more variation in the female median lifespan. The mortality analysis suggests that the general trend despite the lack of significance was that experimental flies had an increased initial rate of mortality but decreased mortality trajectory rate, except the $w^{Dahomey}$ males, where the opposite was true for both cases.

To see if there was any statistical relationship between dAKT reduction and lifespan in males and females from both backgrounds, a linear regression model was fitted to the data with least squares. This was then used to predict lifespan with the level of dAKT kinase activity (**Figure 6.3.6**). Interestingly, only a significant negative linear relationship

was found in $w^{Dahomey}$ females for lifespan and dAKT activity, no such relationship was found in either males or in flies from the w^{1118} background.

			Baseline (α)			Rate (β)		
Background	**Sex**	**Genotype**	α	**LCI**	**UCI**	β	**LCI**	**UCI**
w^{1118}	M	$dAkt^{5F3}$/-	0.00011	0.00005	0.00025	0.09753	0.08551	0.11122
		$dAkt^{6W2}$/-	0.00025	0.00012	0.00054	0.08822	0.07668	0.10149
		$dAkt^{152}$/-	0.00027	0.00013	0.00057	0.08655	0.07526	0.09954
		+/-	0.00007	0.00003	0.00021	0.10954	0.09422	0.12736
		$dAkt^{5F3}$/+	0.00006	0.00002	0.00017	0.09914	0.08544	0.11504
		$dAkt^{6W2}$/+	0.00011	0.00004	0.00037	0.10164	0.08456	0.12218
		$dAkt^{152}$/+	0.00021	0.00009	0.00050	0.09403	0.08105	0.10908
		WT	0.00008	0.00003	0.00020	0.10999	0.09597	0.12605
	F	$dAkt^{5F3}$/-	0.00004	0.00001	0.00011	0.12804	0.11243	0.14582
		$dAkt^{6W2}$/-	**0.00016**	0.00007	0.00036	**0.09326**	0.08147	0.10677
		$dAkt^{152}$/-	0.00012	0.00005	0.00028	0.10730	0.09333	0.12337
		+/-	0.00000	0.00000	0.00003	0.15165	0.13313	0.17276
		$dAkt^{5F3}$/+	0.00002	0.00000	0.00006	0.12499	0.11014	0.14184
		$dAkt^{6W2}$/+	0.00003	0.00000	0.00012	0.12357	0.10500	0.14542
		$dAkt^{152}$/+	0.00009	0.00003	0.00023	0.12044	0.10424	0.13915
		WT	0.00002	0.00000	0.00006	0.13315	0.11745	0.15096
$w^{Dahomey}$	M	$dAkt^{5F3}$/-	**0.00003**	0.00001	0.00011	0.13448	0.11293	0.16015
		$dAkt^{6W2}$/-	**0.00000**	0.00000	0.00003	**0.15858**	0.13004	0.19339
		$dAkt^{152}$/-	0.00022	0.00004	0.00120	0.10238	0.07621	0.13754
		+/-	**0.00001**	0.00000	0.00006	**0.14500**	0.11855	0.17735
		$dAkt^{5F3}$/+	0.00003	0.00001	0.00014	0.12117	0.10107	0.14525
		$dAkt^{6W2}$/+	0.00018	0.00007	0.00049	0.09824	0.08312	0.11611
		$dAkt^{152}$/+	0.00051	0.00022	0.00120	0.07756	0.06459	0.09314
		WT	0.00028	0.00011	0.00071	0.09767	0.08224	0.11600
	F	$dAkt^{5F3}$/-	**0.00211**	0.00119	0.00374	**0.05360**	0.04443	0.06467
		$dAkt^{6W2}$/-	0.00024	0.00010	0.00056	0.08013	0.06839	0.09389
		$dAkt^{152}$/-	0.00062	0.00028	0.00137	0.07026	0.05849	0.08440
		+/-	0.00021	0.00008	0.00057	0.09197	0.07679	0.11014
		$dAkt^{5F3}$/+	0.00086	0.00037	0.00199	0.07427	0.06043	0.09127
		$dAkt^{6W2}$/+	0.00104	0.00052	0.00210	0.07120	0.05942	0.08533
		$dAkt^{152}$/+	**0.00202**	0.00106	0.00385	**0.05957**	0.04846	0.07323
		WT	0.00037	0.00016	0.00087	0.09166	0.07769	0.10813

Table 6.2 Parameters of the Gompertz model for *dAkt* mutants.
Values were estimated by using maximum likelihood procedures in *Winmodest* (Pletcher, 1999). **Bold** font denotes the upper or lower confidence interval of the value lies outside the upper or lower confidence intervals of the wild-type.

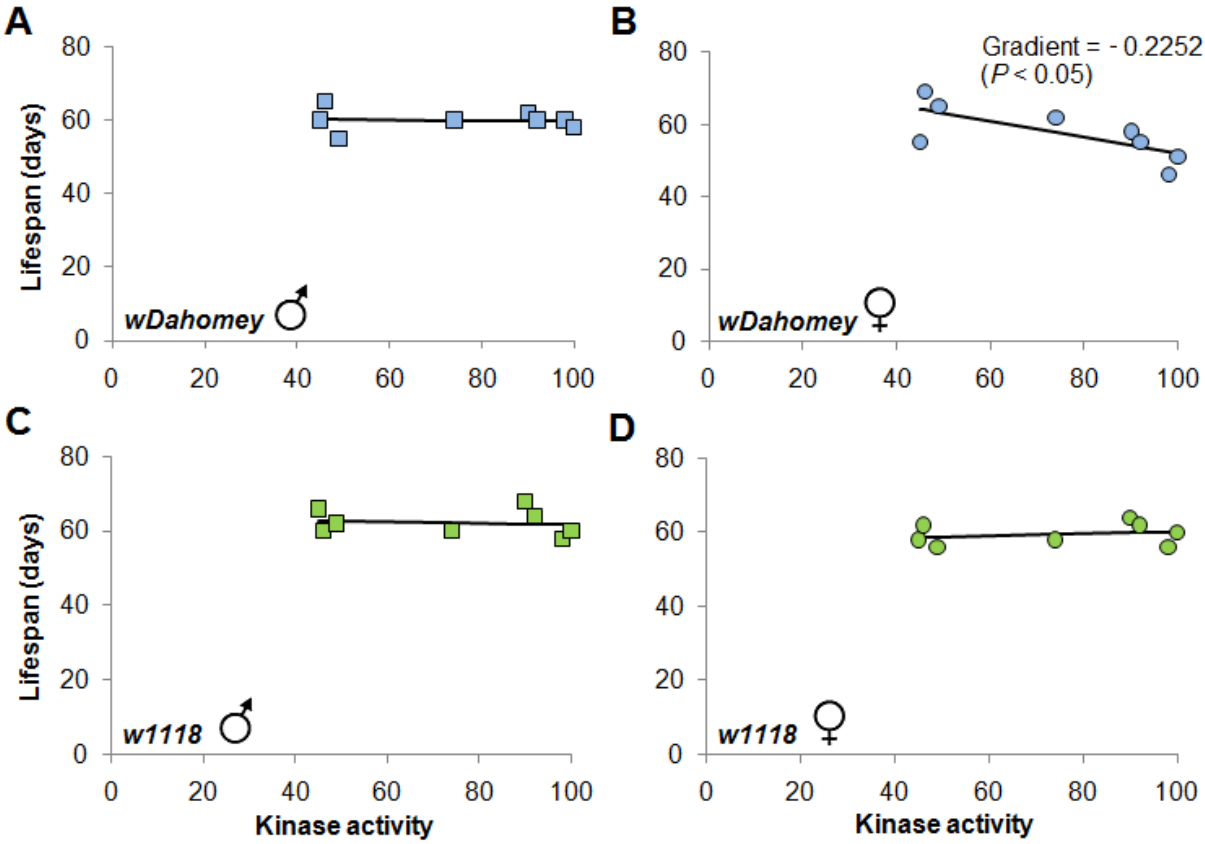

Figure 6.3.6 Kinase activity (IIS) and lifespan.
The kinase activity of each genotype and its respective lifespan from **Table 6.1** was analysed by linear regression**.** This revealed only a significant relationship between the level of IIS and lifespan for females in the $w^{Dahomey}$ background (panel B). This suggests that lifespan extension relies on the both the sex and genetic background the mutation occurs in. For $w^{Dahomey}$ females, extreme low levels of IIS are detrimental to life (Clancy *et al*., 2001), but lifespan may increase at an intermediate level of IIS before decreasing as the level of IIS continues to increase. However, for males and in the w^{1118} background, no such response was observed. Points represent observed male (square) and female (circle) values, whereas lines represent the predicted slope and intercept from the analysis.

Measurements of lifetime reproductive output were also measured and were found to be significantly reduced in mated females compared to controls in both $w^{Dahomey}$ and w^{1118} backgrounds (**Figure 6.3.7**). Fecundity correlated with the mass of the flies and thus the level of signalling, with the most reduced in size displaying the greatest reduction in fecundity. The fecundity of mutant heterozygotes was also mildly lowered.

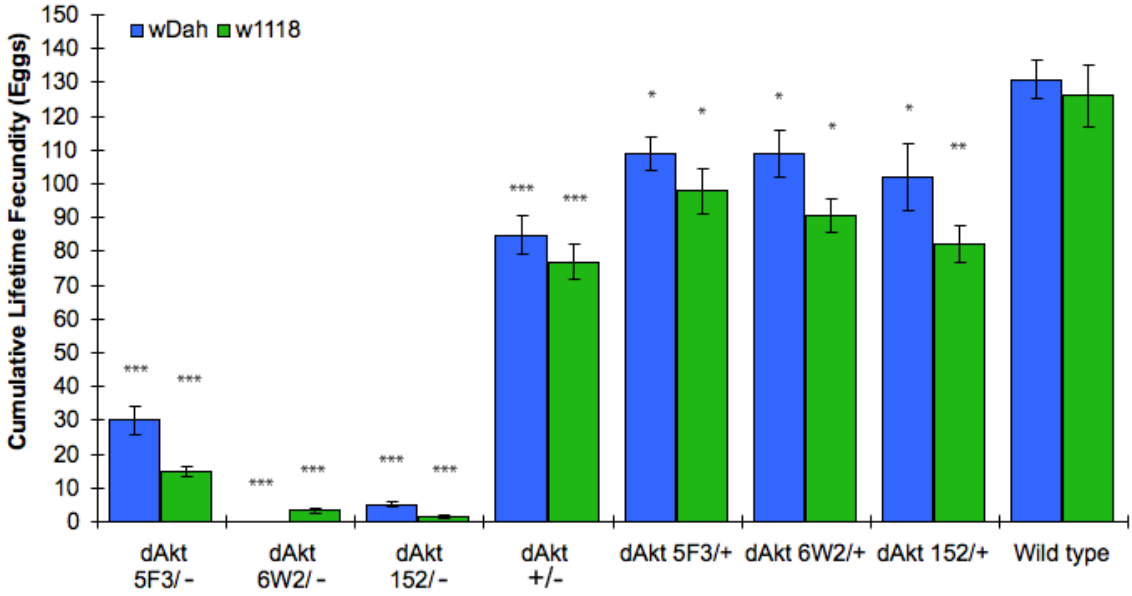

Figure 6.3.7 Comparison of lifetime fecundity in the genotypes in both backgrounds.
Each genotype was raised in parallel under the same conditions and assayed on 1x SY for lifespan. Bars: index of lifetime fecundity ± standard error of mean. Probability that values are identical to wild-type: * = P<0.05; ** = P<0.005; *** = P< 0.0001 (Student's t-test).

6.3.4. Reduced dAKT activity and stress resistance

Resistance to environmental stress is often positively correlated with lifespan in a range of organisms. Selection experiments in *D. melanogaster* for increased longevity generated flies that were not only long-lived compared to control lines but were also starvation and desiccation resistant (Rose, 1984). *Vice versa*, selection for increased starvation and desiccation resistance resulted in flies that were both stress resistant and long-lived (Rose *et al*., 1992; Chippindale *et al*., 1993). Enhanced resistance to stress is often associated with reduced IIS (Honda and Honda, 2002). *C. elegans* with reductions in IIS were also resistant to heat shock, ultraviolet (UV) light, hydrogen peroxide (H_2O_2) and paraquat (oxidative stress) (Larsen, 1993; Lithgow *et al*., 1995; Guarente and Kenyon, 2000; Houthoofd *et al*., 2005), while in mammalian cells, various stresses caused FOXO3a to relocate to the nucleus where SIRT1 deacetylation occurs. This resulted in FOXO-induced oxidative stress resistance and increased DNA repair (Giannakou and Partridge, 2004). Similarly, insulin receptor knock-out mice had extended lifespan and increased resistance to oxidative stress (Holzenberger *et al*., 2003), suggesting that this correlation is conserved in mammals. Reducing IIS signalling in fruit flies also increased resistance to starvation (Clancy *et al*., 2001) and increased levels of superoxide dismutase (SOD), a free

radical defence mechanism (Tatar *et al*., 2001). Flies in which the MNCs were ablated were long-lived and also resistant to starvation and oxidative stress (Broughton *et al*., 2005), while over-expression of dFOXO in the head fat-body also inferred oxidative stress resistance.

Here, *dAkt* mutant females were found to have enhanced resistance against paraquat exposure (oxidative stress) as they exhibited increased survival during paraquat treatment compared to controls in w^{1118} and $w^{Dahomey}$, whereas males did not exhibit any paraquat survival advantage except in $w^{Dahomey}$; $dAkt^{+/-}$ (**Figure 6.3.8A-D**). Mutant flies subjected to hyperoxia displayed similar increased survival advantages: the majority of female *dAkt* mutants and male $dAkt^{1}$ heterozygotes were observed to exhibit increased resistance to oxidative stress (**Figure 6.3.8E-F**). Additionally, females and males were starvation resistant in the *yw* background, as were male $dAkt^{+/-}$ in the $w^{Dahomey}$ background. Other males and all females in the $w^{Dahomey}$ did not have increased survival against starvation (**Figure 6.3.8G**).

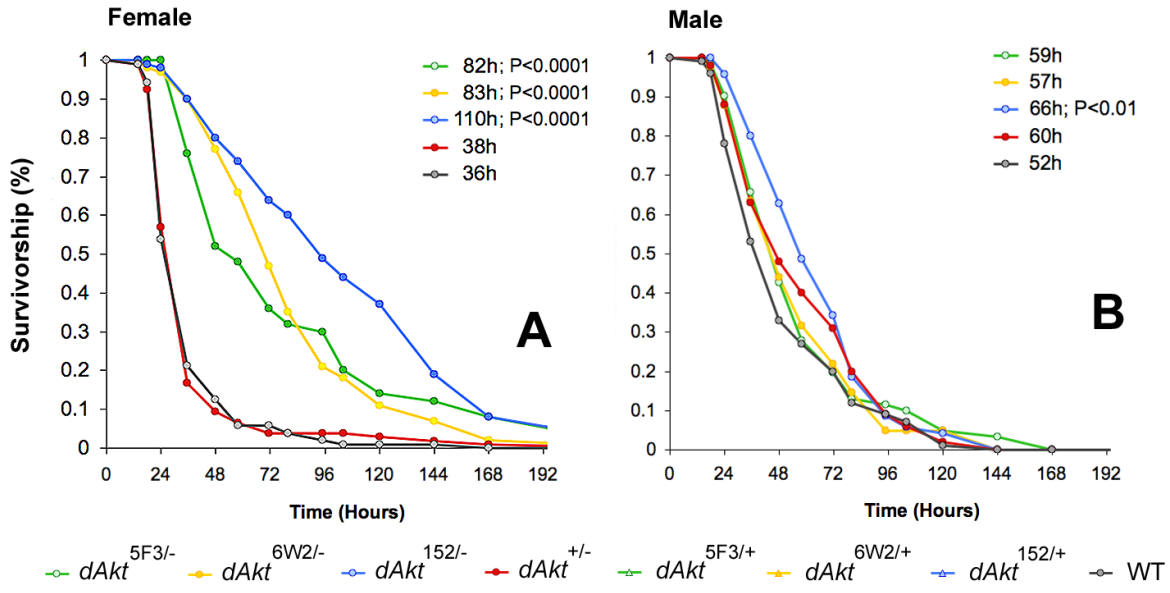

Figure 6.3.8 Effect of *dAkt* reduction on stress resistance.
Each genotype was raised in parallel under the same conditions and assayed at 7 days of age (*N* = 100 in all experiments). The graph legend reports the mean lifespan in days and the probability that survival is identical to wild-type is indicated as a *P*-value (log-rank). Probability that values are identical to wild-type: * = *P*<0.05; ** = *P*<0.001; *** = *P*< 0.0001 (log-rank). Females on the left, males on the right. Paraquat: Flies were assayed on 1xSY containing 20mM paraquat for survival. (**A**) Female lifespan in w^{1118} (**B**) Male lifespan in w^{1118}

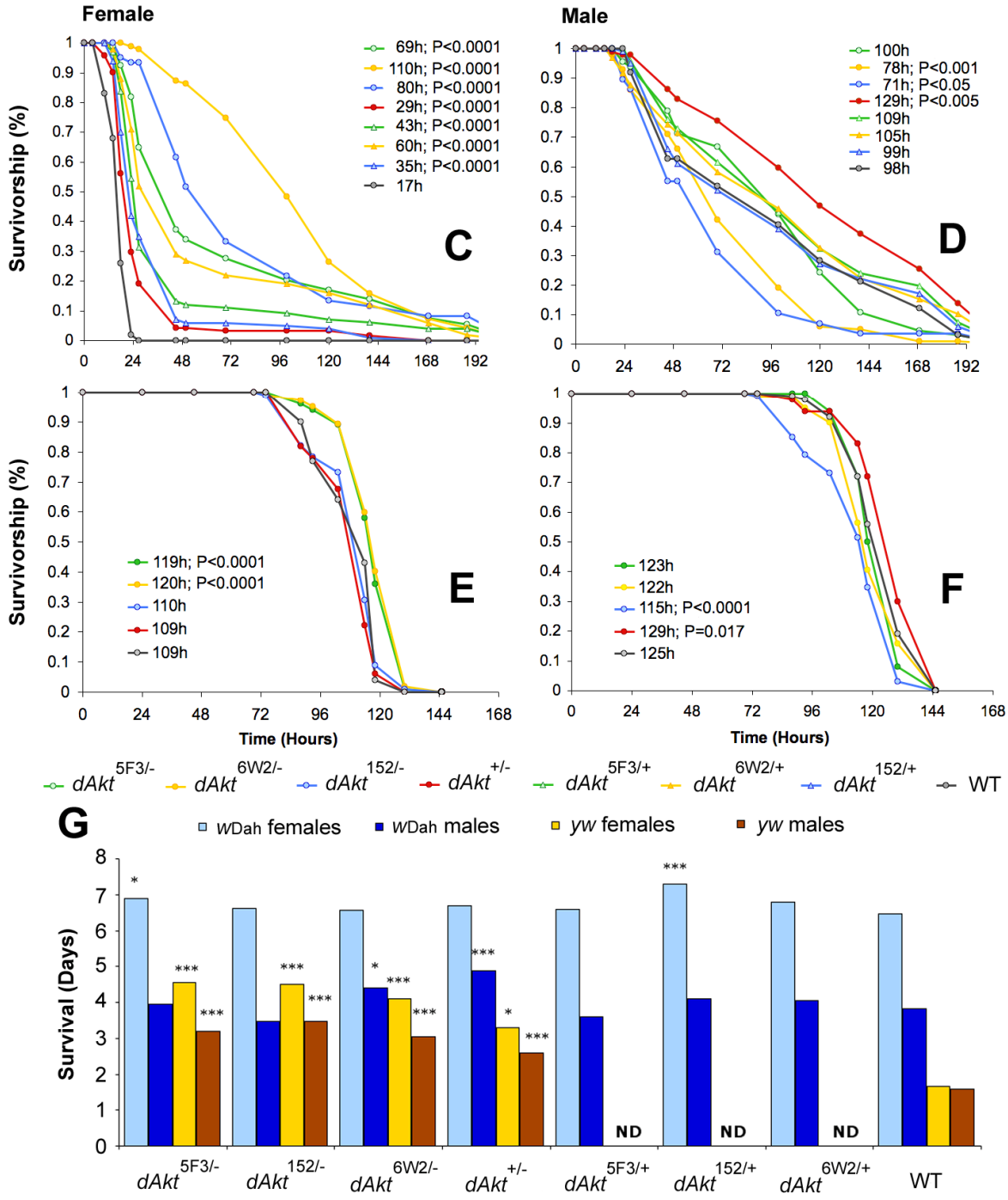

Figure 6.3.8 Effect of *dAkt* reduction on stress resistance (continued).
Paraquat: Flies were assayed on 1xSY containing 20mM paraquat for survival.
(**C**) Female lifespan in $w^{Dahomey}$
(**D**) Male lifespan in $w^{Dahomey}$. Hyperoxia: Flies were assayed on 1xSY in a hyperbaric chamber at 90% oxygen for survival.
(**E**) Female lifespan in $w^{Dahomey}$
(**F**) Male lifespan in $w^{Dahomey}$. Females were also assayed for resistance to starvation on a 1% agar medium from day 7 of life
(**G**) Effect of *dAkt* reduction on starvation resistance. Bars indicate mean survival time. Mean survival in *yw* and white^{Dahomey} male and female flies on 1% agar. Probability that values are identical to wild-type: * = $P<0.05$ and *** = $P< 0.0001$ (log-rank).

6.4. Results: RNA interference of *dPI3K* and *dAkt*

6.4.1. Sufficient IIS knockdown by *Dp110* RNAi and *dAkt* RNAi leads to a reduction in fecundity

Additional to mutant studies of *dAkt*, one can look for similar effects in gene knockdown mutants using the transgenic RNA interference (RNAi) approach (Fire *et al*., 1998; Dietzl *et al*., 2007). Several lines containing UAS-driven inverted repeat transgenes of either the *dAkt* or, the catalytic subunit of dPI3K, *Dp110* (*w;* T1, *w;* T2 and *w;* T3) (Dietzl *et al*., 2007) were obtained and crossed to *daughterless*-GAL4 in order to get ubiquitous constitutive induction of IIS RNAi. Ubiquitous knockdown of either *dAkt* or *Dp110* during development caused lethality in L2 larvae. Therefore, conditional expression of *dAkt*-RNAi and *Dp110*-RNAi was required to bypass developmental lethality as well as to study its impact on ageing exclusively in the adult stage.

Thus, the drug mifepristone (RU486) inducible-GAL4 system, annotated to either P[Switch] (Roman *et al*., 2001) or *GeneSwitch*, *GS* (Osterwalder *et al*., 2001), was used to drive the expression of UAS constructs in defined adult tissues. The inducible but ubiquitously expressed driver *tubGS* and inducible gut/fat-body specific driver P[Switch] S_1106 (shortened here to S_1106) (Poirier *et al*., 2008) were selected. Such a study has been performed before using S_1106 to specifically over-express *dFOXO* in the abdominal fat-body where it was reported to extend lifespan (Giannakou *et al*., 2004; Hwangbo *et al*., 2004).

Here, flies were exposed to two concentrations of RU486 added to the food, 100µM and 200µM. This was a test to see if different concentrations of RU486 taken up by flies would result in different levels of knockdown. Thus, RNAi lines driven by *tubGS* were maintained on food containing RU486 at 2 days after eclosion. After 5 days of adult RNAi induction by mifepristone ingestion, *dAkt* and *Dp110* transcript levels were measured by quantitative RT-PCR where endogenous *dAkt* and *Dp110* expression was reduced to consistently low levels (**Figure 6.4.1A-D**). Induction by 200µM RU486 resulted in a greater knockdown of either the *dAkt* or *Dp110* gene than induction by 100µM RU486. Next, fecundity measurements were taken of RNAi lines driven by both *tubGS* and S_1106 at the two different concentrations. Both driver control lines displayed mild reduction in fecundity when compared to the wild-type, thus all experimental lines were compared to

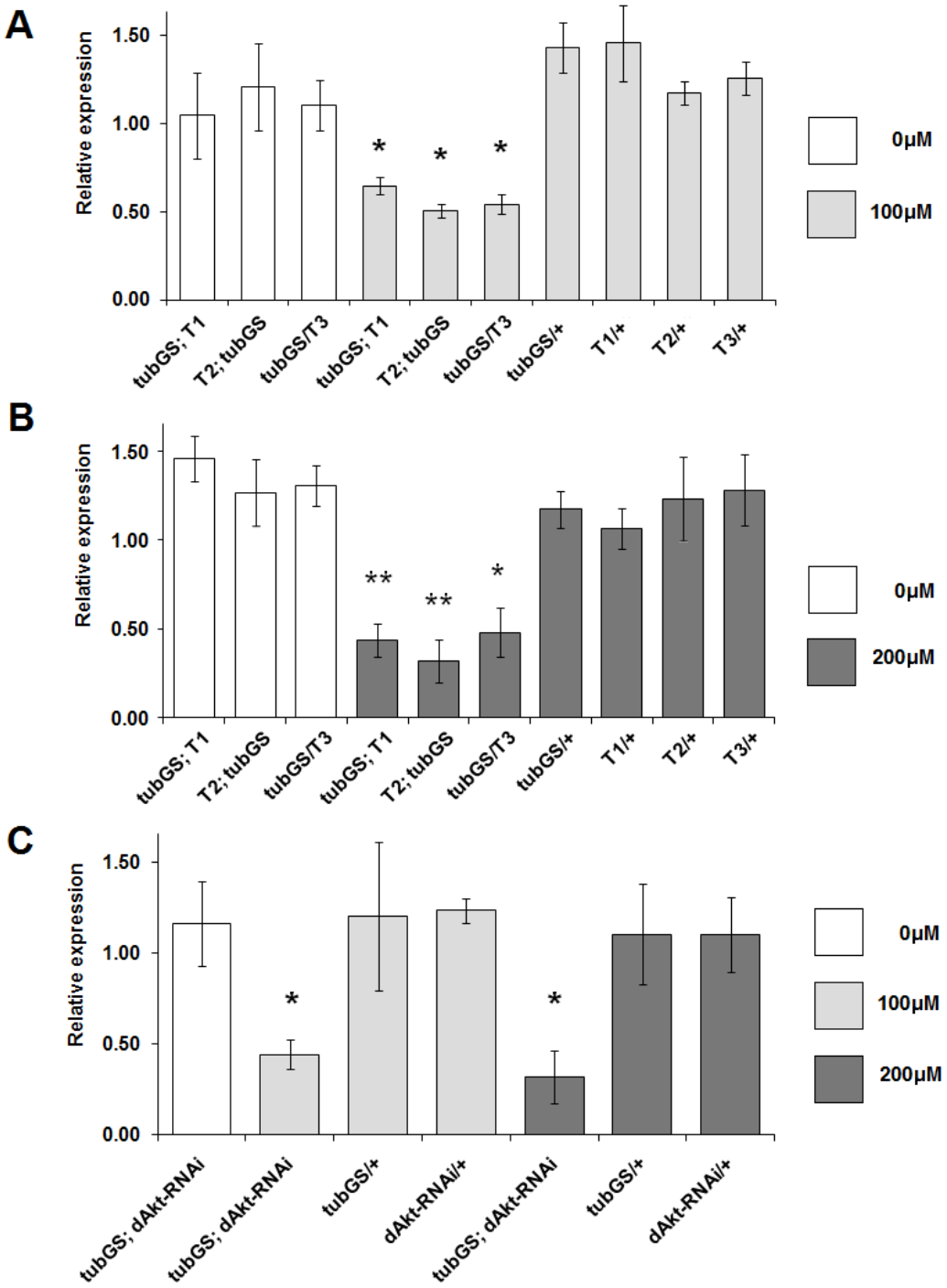

Figure 6.4.1 IIS knockdown by RNAi.
RNAi constructs were driven either ubiquitously (*tubGS*) or in the fat body and gut (S_1106) by RU486 induction via adult fly food. White bars = no RU486 induction, light grey bars = 100µM RU486 and grey bars = 200µM RU486 exposure. The probability that experimental lines are identical to control lines is indicated as: * = $P<0.05$; ** = $P<0.005$; *** = $P<0.0001$ (Wilcoxon). (**A**) The effect of ubiquitous *Dp110*-RNAi induction by 100µM RU486 on expression of *PI3K* measured by RT-PCR. Within each sample, *Dp110* mRNA was normalised by the abundance of *actin*, and abundance expressed relative to the plate average.
(**B**) The effect of ubiquitous *Dp110*-RNAi induction by 200µM RU486 on expression of *PI3K* measured by RT-PCR. Within each sample, *Dp110* mRNA was normalised by the abundance of *actin*, and abundance expressed relative to the plate average.
(**C**) The effect of ubiquitous *dAkt*-RNAi induction by 100µM and 200µM RU486 on expression of *dAkt* measured by RT-PCR. Within each sample, *dAkt* mRNA was normalised by the abundance of *actin*, and abundance expressed relative to the plate average.

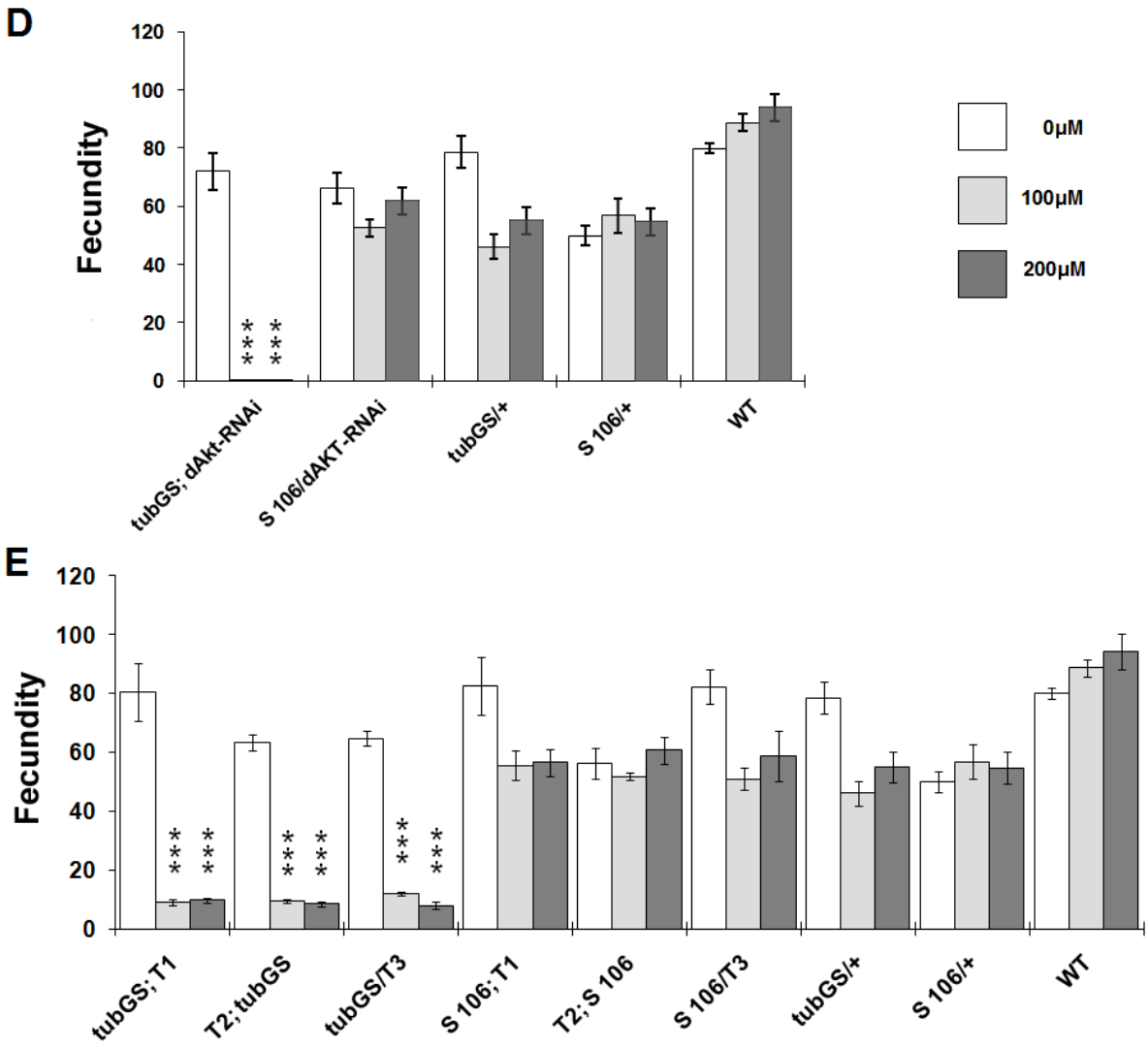

Figure 6.4.1 IIS knockdown by RNAi (continued).
(**D**) Cumulative lifetime fecundity (the number of eggs laid per female) of *dAkt*-RNAi was significantly reduced in *tubGS* driven flies but not when driven by S_1106.
(**E**) Cumulative lifetime fecundity (the number of eggs laid per female) of *Dp110*-RNAi was reduced when expressed ubiquitously but no difference was observed when expressed by S_1106. Data shown are from a single trial in which all fecundity experiments were run simultaneously with the lifespan experiments (**Figure 6.4.2**).

the driver control. *dAkt*-RNAi lines were sterile and *Dp110*-RNAi were significantly reduced when driven by *tubGS* at all RU486 concentrations, while no significant difference was found in any RNAi lines when driven by S_1106.

6.4.2. IIS knockdown in the abdominal fat body results in lifespan extension

All RNAi lines for *dAkt* and *Dp110* were then driven by *tubGS* and S_1106 at both RU486 concentrations and assessed for their survival. No significant effect on lifespan was

observed when *dAkt* was ubiquitously knocked down at 100µM and 200µM RU486 when compared to its appropriate *tubGS* driver control (**Figure 6.4.2A**). Furthermore, experimental flies were significantly shorter lived compared to the wild type (100µM: $P < 0.01$ and 200µM: $P < 0.0001$). However, gut and abdominal fat-body specific knock-down of *dAkt* at 200µM RU486 resulted in significant lifespan extension to 67 days median lifespan compared to the *S_1106* driver control (54 days, $P < 0.0005$) and the wild type (64 days, $P < 0.05$). Experimental lines driven by 100µM RU486 also displayed increased survival when compared to the *S_1106* driver (from 58 to 67 days, $P < 0.0001$) but not the wild type (**Figure 6.4.2B**).

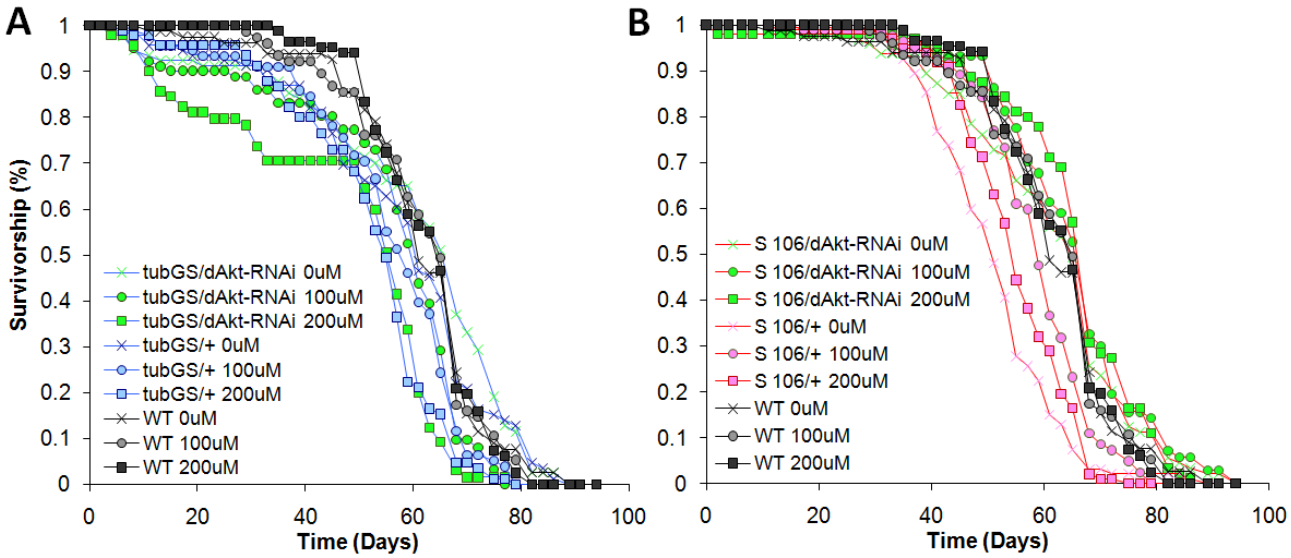

Figure 6.4.2 Survival in experimental RNAi lines.
dAkt-RNAi and *Dp110*-RNAi were driven by either *tubGS* (blue line) or *S_1106* (red line). Wild-type flies are denoted by black lines. Green markers represent driver and RNAi crossing. Blue markers represent *tubGS* driver lines. Pink markers represent *S_1106* driver lines. Flies were raised in standard larval density on 1xSYBrewers yeast. 2 days after eclosion, females were transferred to vials containing either 100µM (circles) RU486, 200µM (square) RU486 or no (cross) RU486 food. Flies were scored for survival and transferred to fresh food vials every 2 days.
(**A**) *dAkt*-RNAi line was driven by *tubGS* driver on two RU486 concentrations. No significant extension of lifespan was observed in experimental lines when compared to the driver lines.
(**B**) *dAkt*-RNAi line was driven by *S_1106* driver on two RU486 concentrations. Significant extension of lifespan was observed in experimental lines when compared to the driver lines.

Ubiquitous knockdown of *Dp110* resulted in extended lifespan in one line (T2) but not in two other lines (T1 and T3), whereas abdominal fat body specific knockdown of *Dp110* increased survival in all lines. Increased survival was only observed when experimental lines were compared to their respective drivers at their respective RU486 concentrations,

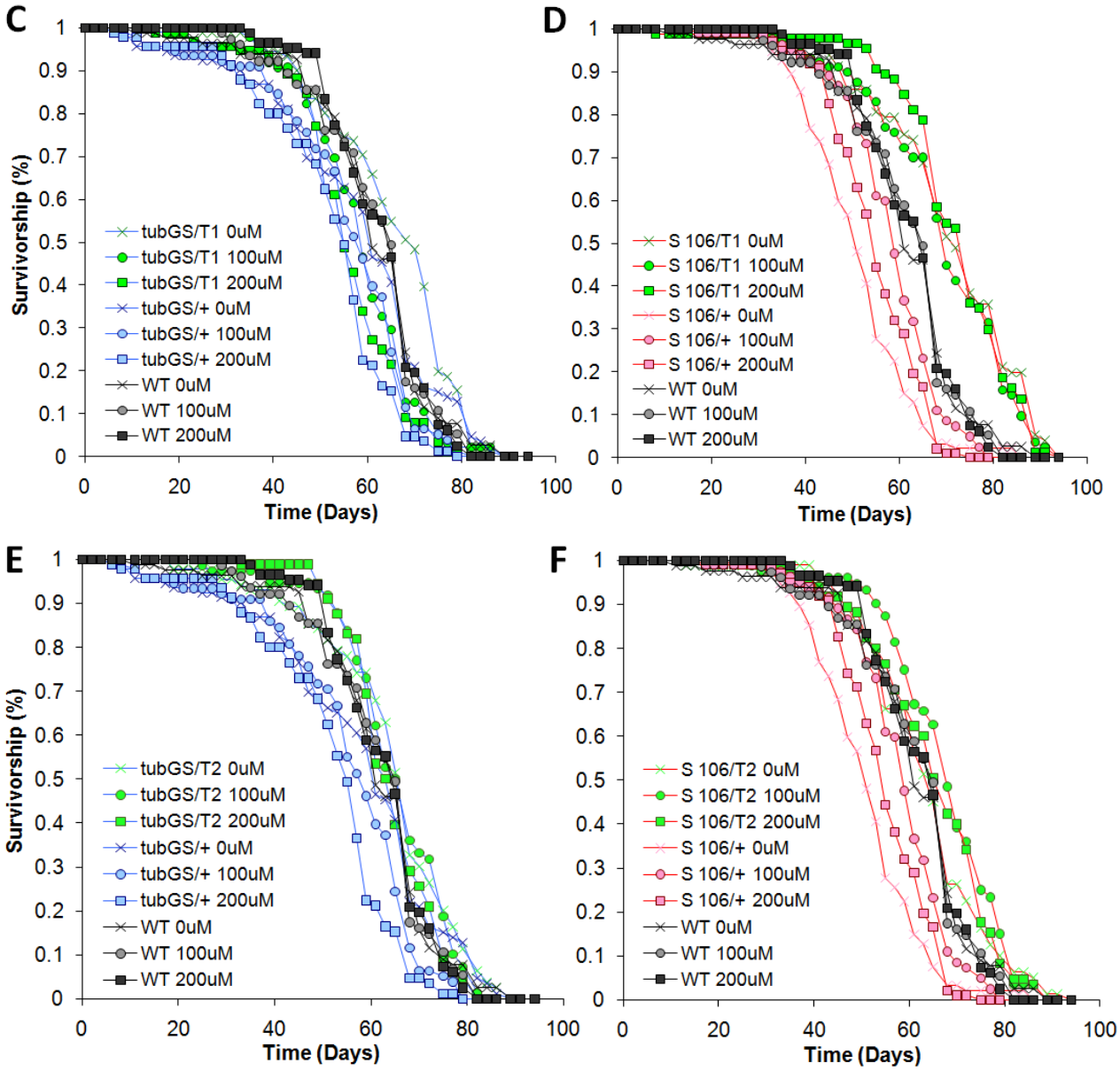

Figure 6.4.2 Survival in experimental RNAi lines (continued).
(**C**) *Dp110*-RNAi line (T1) was driven by *tubGS* driver on two RU486 concentrations. No significant extension of lifespan was observed in experimental lines when compared to the driver controls.
(**D**) *Dp110*-RNAi line (T1) was driven by *S*$_1$*106* driver on two RU486 concentrations. Significant extension of lifespan was observed in experimental lines when compared to the driver controls.
(**E**) *Dp110*-RNAi line (T2) was driven by *tubGS* driver on two RU486 concentrations. Significant extension of lifespan was observed in experimental lines when compared to the driver controls.
(**F**) *Dp110*-RNAi line (T2) was driven by *S*$_1$*106* driver on two RU486 concentrations. Significant extension of lifespan was observed in experimental lines when compared to the driver controls.

no extension of lifespan was observed in any case when compared to the wild type. At 100µM and 200µM RU486 induction, the lifespan of experimental T1 RNAi line did not significantly differ compared to the *tubGS* driver control or the wild type. However, experimental lines not fed RU486 were found to be the longest lived (69 day median lifespan, $P < 0.0001$) of all treatments (**Figure 6.4.2C**). When the T1 line was driven by

S₁106, lifespan was found to be significantly extended at both RU486 concentrations (100µM: 69 days, $P < 0.0001$ and 200µM: 74 days, $P < 0.05$) compared to its driver control (58 and 54 days, respectively) (**Figure 6.4.2D**). The survival of experimental *Dp110* RNAi line T2 was found to be significantly extended compared to driver controls when driven by both *tubGS* and *S₁106* drivers at all concentrations of RU486 ($P < 0.0005$) (**Figure 6.4.2E-F**).

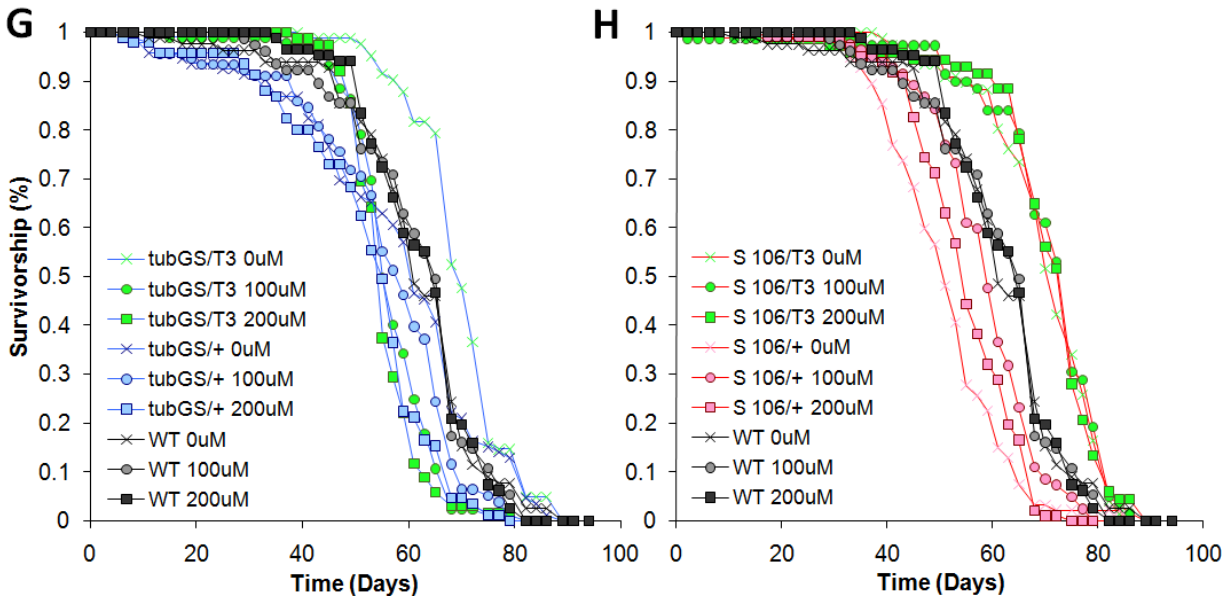

Figure 6.4.2 Survival in experimental RNAi lines (continued).
(**G**) *Dp110*-RNAi line (T3) was driven by *tubGS* driver on two RU486 concentrations. No significant extension of lifespan was observed in experimental lines when compared to the driver control. Although at 0µM RU486, lifespan of experimental lines were found to be significantly increased.
(**H**) *Dp110*-RNAi line (T3) was driven by *S₁106* driver on two RU486 concentrations. Significant extension of lifespan was observed in experimental lines when compared to the driver control.

Experimental *Dp110* RNAi line T3 did not exhibit extended lifespan compared to driver controls when ubiquitously induced by *tubGS* drivers at both RU486 concentrations (**Figure 6.4.2G**). However, when the RNAi lines was induced by the *S₁106* driver, median survival was increased significantly compared to the driver (100µM: from 58 to 74 days, $P < 0.0001$ and 200µM: from 54 to 74 days, $P < 0.05$) (**Figure 6.4.2H**).

Finally it is worth noting that in all experiments, the *tubGS/+* and *S₁106/+* driver controls were all observed to be shorter lived than the wild type controls. However, the reduction in lifespan was only significant in driver controls induced at 200µM RU486.

6.4.3. *Dp110* and *dAkt* RNAi lines display resistance to oxidative stress

To assess whether the resistance to oxidative stress was still apparent with IIS disruption, females subjected to IIS RNAi were measured for paraquat survival. Resistance to paraquat was observed when all the RNAi constructs were driven by *tubGS* on both RU486 concentrations, except in one *Dp110*-RNAi line (T3) on 100µM RU486 induction. *dAkt* expression by *$S_1$106* was found to extend survival against paraquat at 200µM RU486 induction but not at 100µM RU486 induction. *$S_1$106* driven *Dp110*-RNAi lines also showed paraquat resistance at both concentrations of RU486 except in one line (T3), which was found to be shorter lived than the driver control for both RU486 concentrations (**Figure 6.4.3**). Finally no resistance to starvation was observed in experimental lines driven by *tubGS* at 200µM RU486 when compared to either wild type or *tubGS* driver controls (**Figure 6.4.4**).

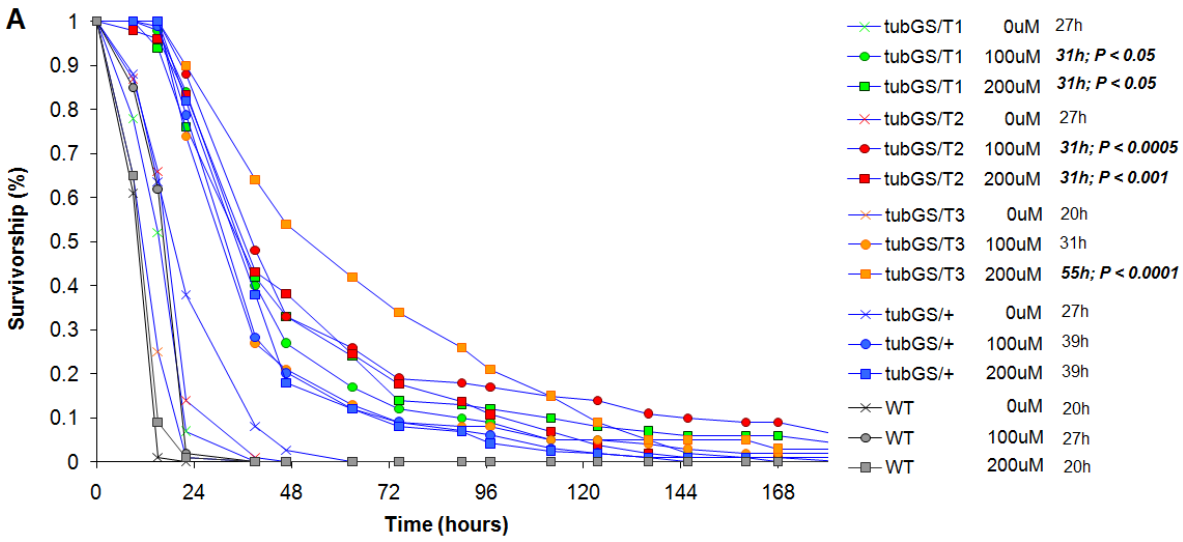

Figure 6.4.3 Survival to paraquat in experimental RNAi lines.
Dp110 or dAkt-RNAi lines in the *w*^{Dahomey} background were driven by either *tubGS* (blue line) or *$S_1$106* (red line). Wild-type flies are denoted by black lines. Colour of markers: *dAkt-RNAi* = brown, *Dp110-RNAi* (T1) = green, *Dp110-RNAi* (T2) = red, *Dp110-RNAi* (T3) = orange, *tubGS* driver control = blue and wild-type (WT) = black/grey.
Flies were raised in standard larval density on 1xSYBrewers yeast. 2 days after eclosion, females were transferred to vials containing either 100µM (circles) RU486, 200µM (square) RU486 or no (cross) RU486 food. After 5 days of induction, day 7 of lifespan, flies were transferred to food containing 20mM paraquat and scored for survival. The probability that the median lifespan is not significantly different to the driver control is displayed in the legend. Significantly different lifespan are in bold font.
(**A**) Resistance to paraquat was observed in experimental *Dp110-RNAi* flies when compared to the RU486 induced driver control (*tubGAL4/+*), except in T3 line at 100µM RU486 induction.

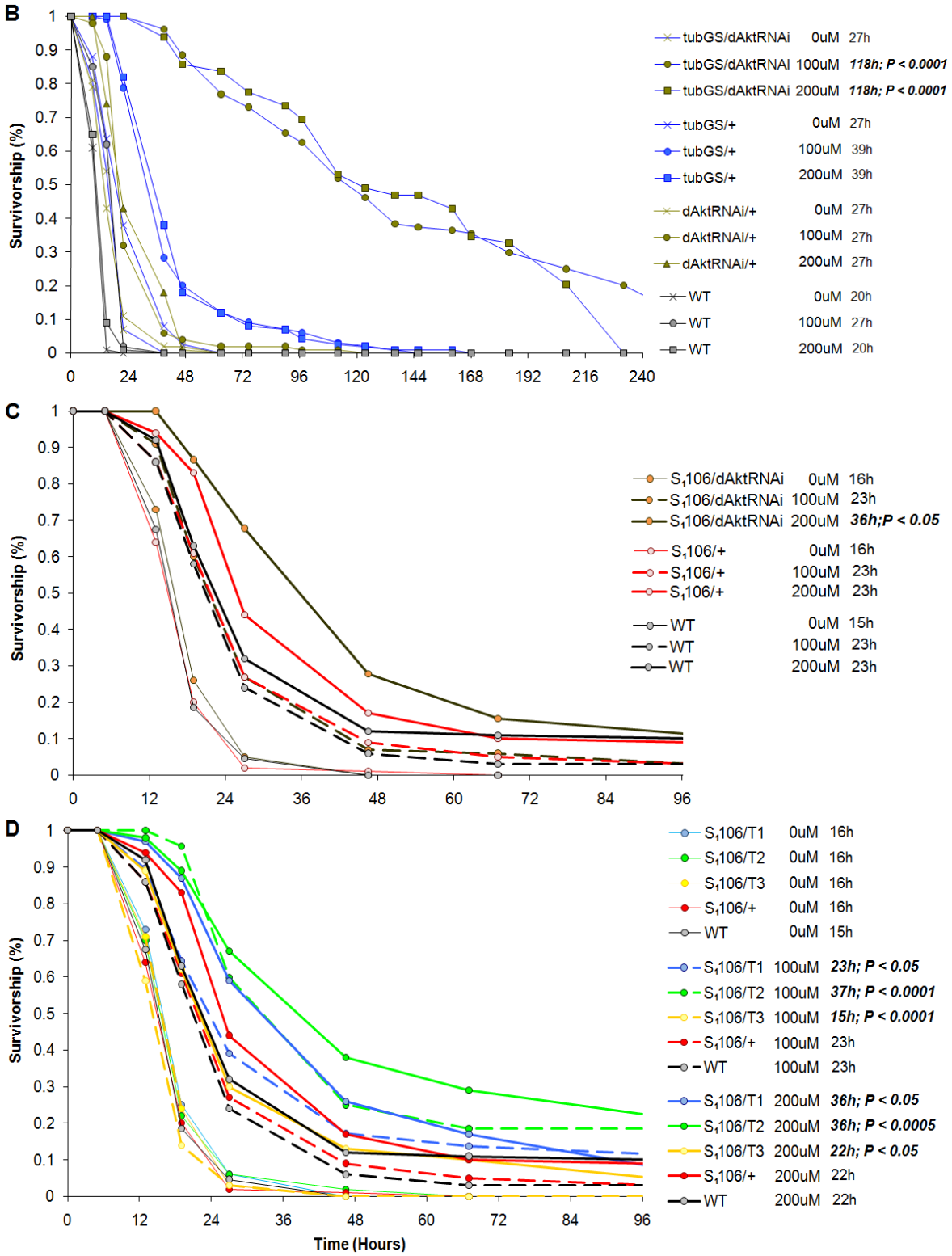

Figure 6.4.3 Survival to paraquat in experimental RNAi lines (continued).
(**B**) Resistance to paraquat was observed in experimental *dAkt-RNAi* flies when compared to the RU486 induced driver control (*tubGAL4/+*) at both levels of RU486 induction.
(**C**) Resistance to paraquat was observed in *$S_1$106* driven *dAkt*-RNAi lines at 200µM RU486 induction but not 100µM RU486 induction.
(**D**) Significantly increased resistance to paraquat was observed in *Dp110*-RNAi (T1 and T2) lines compared to the induced *$S_1$106/+* driver control at both RU486 concentrations. T3 RNAi lines were significantly shorter lived in both.

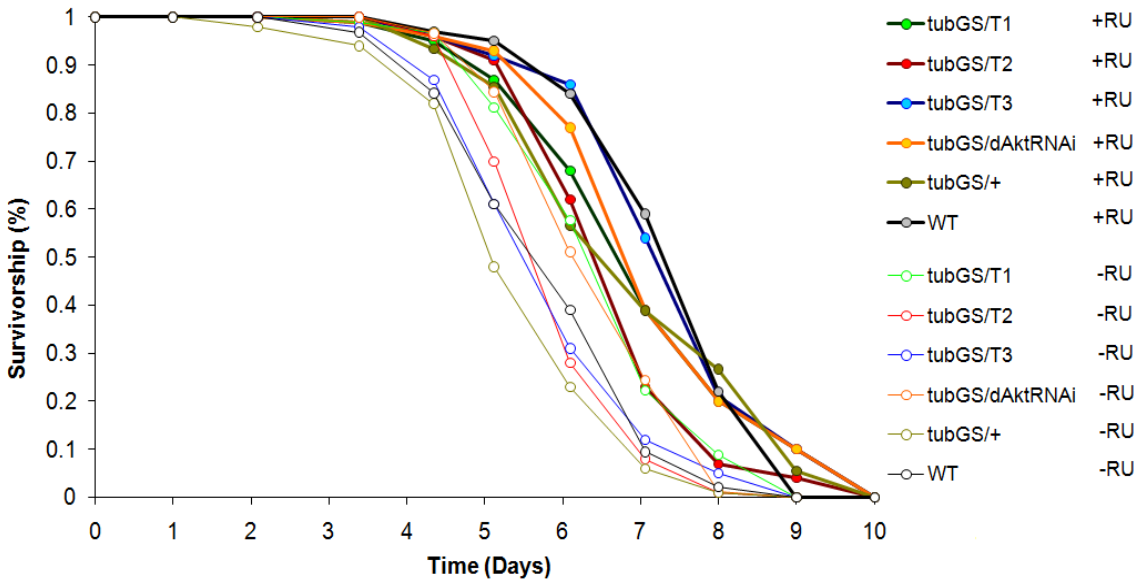

Figure 6.4.4 Survival to starvation in experimental RNAi lines.
Dp110 or dAkt-RNAi lines in the $w^{Dahomey}$ background were driven by *tubGS*. Wild-type flies are denoted by black lines. Flies were raised in standard larval density on 1xSYBrewers yeast. 2 days after eclosion, females were transferred to food vials containing 200µM RU486 or no RU486. After 5 days of induction, day 7 of lifespan, flies were transferred to 1% agar containing 200µM RU486 (closed circles) or no RU486 (open circles) and scored for survival. Colour of markers: *dAkt-RNAi* = orange, *Dp110-RNAi* (T1) = green, *Dp110-RNAi* (T2) = red, *Dp110-RNAi* (T3) = blue, *tubGS* driver control = brown and wild-type (WT) = black/grey. No significant resistance to starvation was observed in any experimental lines.

6.5. Discussion

6.5.1. Extension of *Drosophila* lifespan by reduction of dAKT activity

Several genetic interventions in the insulin/ insulin-like signalling (IIS) pathway have resulted in increased longevity in a variety of species, which include worms (Johnson, 1990; Kenyon *et al*., 1993; Kimura *et al*., 1997; Tissenbaum and Ruvkun, 1998) and mice (Bluher *et al*., 2003; Holzenberger *et al*., 2003). In fruit flies, studies have provided robust lifespan extension for the manipulations to the IIS receptor and its substrate, *chico* (Clancy *et al*., 2001; Tatar *et al*., 2001). Furthermore, over-expression of dFOXO in fat-body tissue, a downstream target of the IIS pathway also extends lifespan (Giannakou *et al*., 2004; Hwangbo *et al*., 2004). However, no extension of lifespan has been observed in dPI3K mutants or dAKT mutants. Thus, it is unknown whether the lifespan signal from either the receptor or *chico* is transmitted to FOXO via the dPI3K/dAKT pathway or an alternate pathway. In this chapter, extension of *Drosophila* lifespan by reduction of dAKT activity was shown to be possible supporting the view that these intermediaries play a

role in lifespan determination. However, for a full confirmation of the role of dAKT, *dFOXO* and *dAkt* double mutants will have to be generated to test whether the effects observed here are indeed dFOXO dependent.

Although lifespan extension occurs in *dAkt* mutants, that these mutations increase lifespan yet are not the wild-type allele suggests they impose some fitness cost. Indeed, a reduction in both body size and fecundity was observed in *dAkt* mutants, results that are similar to studies involving *Inr* and *chico* mutants (Clancy *et al*., 2001; Tatar *et al*., 2001). Two theories have attempted to explain these observations: one theory is that an organism is often faced with limited resources and costly processes, such as reproduction, compete for these resources with other processes such as repair or growth. Thus, a trade-off occurs where the organism must strategise its resources between reproduction, growth and lifespan (Kirkwood, 1977). Another theory proposes that reproduction is a costly process because the act, either through mating or egg production, causes damage that increases mortality early in life (Sgro and Partridge, 1999); and thus, reducing fecundity results in increased longevity. However, this has not been found under all circumstances. Ablation of germ line tissue in male and female *D. melanogaster* did not extend lifespan (Barnes *et al*., 2006), and flies that have been sterilised by either X-ray irradiation or ovo^{D1} mutation show a normal response to dietary restriction, an intervention that has been thought to affect ageing partly through lowered fecundity (Mair *et al*., 2004). This is reminiscent of results in *C. elegans*, where adults with induced sterility from ablation of gonad precursor cells during their juvenile stage were not longer lived (Hsin and Kenyon, 1999). Interestingly, in the same study, ablating the precursors of the germ line cells resulted in an adult with significantly extended longevity (Hsin and Kenyon, 1999). Therefore it has been postulated that it is not sterility *per se* that alters longevity, but rather the presence or absence of specific reproductive signals; and for lifespan extension to occur the upstream somatic gonad signalling must be eliminated before germ line stem cell proliferation (Arantes-Oliveira *et al*., 2002).

The longevity of the *dAkt* mutants described here may not be caused directly by changes in physiology (body size and metabolic stores) or reproduction. As in other examples of fly lifespan extension by IIS disruption (Tatar *et al*., 2001; Garofalo, 2002; Hwangbo *et al*., 2004), increased levels of lipids and other metabolic stores such as trehalose (Broughton *et al*., 2005) were found in *dAkt* mutants. The physiological change

recorded here may only be a side-effect of reduced IIS but the role of increased metabolites in longevity, if any, is still undetermined. The trade-off between fecundity/growth and lifespan may not always be obligate. There are many documented cases of increased survival without reduction in fecundity or body size. *chico* heterozygotes were reported to be longer lived but had a wild-type body size and near normal fecundity (Clancy *et al*., 2001), as were long-lived flies with head fat-body over-expression of dFOXO (Hwangbo *et al*., 2004). In this chapter, some dAkt and PI3K knockdown flies (dAkt-RNAi, T1 and T3) displayed reduced fecundity without extended lifespan when driven ubiquitously, suggesting that signalling pathways for lifespan and fecundity may indeed be separate.

Knockdown of either *Dp110* or *dAkt* gene expression in the abdominal fat body and ubiquitous knockdown *dAkt* gene expression (T2) led to increased survival when compared to the driver control but not when compared to the wild type control. This supports the data from the hypomorphs which suggest that disruption of the dAkt/PI3K pathway does result in lifespan extension. Furthermore, the robust extension of lifespan that resulted from IIS gene knockdown in the abdominal fat body supports the view that dFOXO up-regulation is tissue-specific and important in the adult fat body (Giannakou *et al.,* 2004; Giannakou *et al.*, 2008).

Interestingly, the driver controls were consistently observed to be shorter lived than than wild type controls, with further shortening with increasing RU486 concentration. This suggests that induction of Gene-Switch GAL4 may be detrimental to the health and/ or lifespan of flies. Thus, any lifespan benefits – bestowed from IIS knockdown in experimental RNAi flies – may be masked by the side-effects inherent to the Gene-Switch system. Indeed, induced experimental RNAi lines were not observed to be significantly longer lived than wild type controls but were longer lived than the driver lines alone. Additionally, it has been reported that many Gene-Switch fly strains give leaky expression, without RU486, during development and/or during adulthood (Poirier *et al.*, 2008), thus experimental lines without RU486 induction reported here may be experiencing mild gene knockdown, although this was not detected by quantitative RT-PCR. This may explain the increased longevity observed in RNAi experimental lines not fed RU486.

6.5.2. A subtle level of IIS reduction is required for lifespan extension

The role of the dPI3K/dAkt pathway in fly growth is better documented than its role in lifespan. PI3K and AKT are known to be responsible for controlling a number of functions in the cell (**Figure 6.5.1**), and disruption of either not only affects cell growth but also the viability of the cell. Of the few studies that disrupted *dPI3K* or *dAkt*, none have reported extension of lifespan. This may be because homozygous nulls mutations of either *dPI3K* or *dAkt* resulted in death during larval development (Staveley *et al*., 1998; Weinkove *et al*., 1999). Indeed, it is seen in this chapter that ubiquitously driven RNAi during development against either one of these genes resulted in L2 arrested development. Furthermore, in the same study which discovered lifespan extension in $chico^{1}$ mutants (Clancy *et al*., 2001), lifespan was also measured for a hypomorphic mutation of *dAkt* and null mutations for the catalytic (*Dp110*) and adaptor (*p60*) PI3K subunits for which no difference in lifespan was found. $dAkt^{3}$ (a.k.a. PKB^{3}) flies were reported to contain a hypomorphic mutation which resulted in viable dwarf adults that had a 30% reduction in dAKT activity (Stocker *et al*., 2002) and also a decreased lifespan (Clancy *et al*., 2001). The reasons for this shorter lifespan are not known, but it is possible that precise quantitative modulations of IIS are required to extend lifespan.

In this chapter, three new dAkt hypomorph mutants were characterised. The reduction in kinase activity of these hypomorphs was weaker and thus may not shorten lifespan as the $dAkt^{3}$ mutation did. These were backcrossed into two different genetic backgrounds and lifespan was measured when merged with the null $dAkt^{1}$ mutation (Staveley *et al*., 1998). Despite mild but significant extensions of lifespan, it was clear that both genetic background and sex played a role in whether lifespan extension existed. Female and male hypomorphs from the $w^{Dahomey}$ background were the only flies to have consistent lifespan extension (although extension of lifespan was not as great in males), while there was less robust lifespan extension in flies from the w^{1118} background. Furthermore, males and female hypomorphs from the $w^{Dahomey}$ background also had the ability to cope with oxidative stress more robustly. Western blot analysis of protein from flies of the w^{1118} background revealed that FOXO phosphorylation levels although lowered were not significantly different between *dAkt* mutants and the wild-type; and thus, may explain

why no significant relationship was found between lifespan and reduced dAKT activity in the same background.

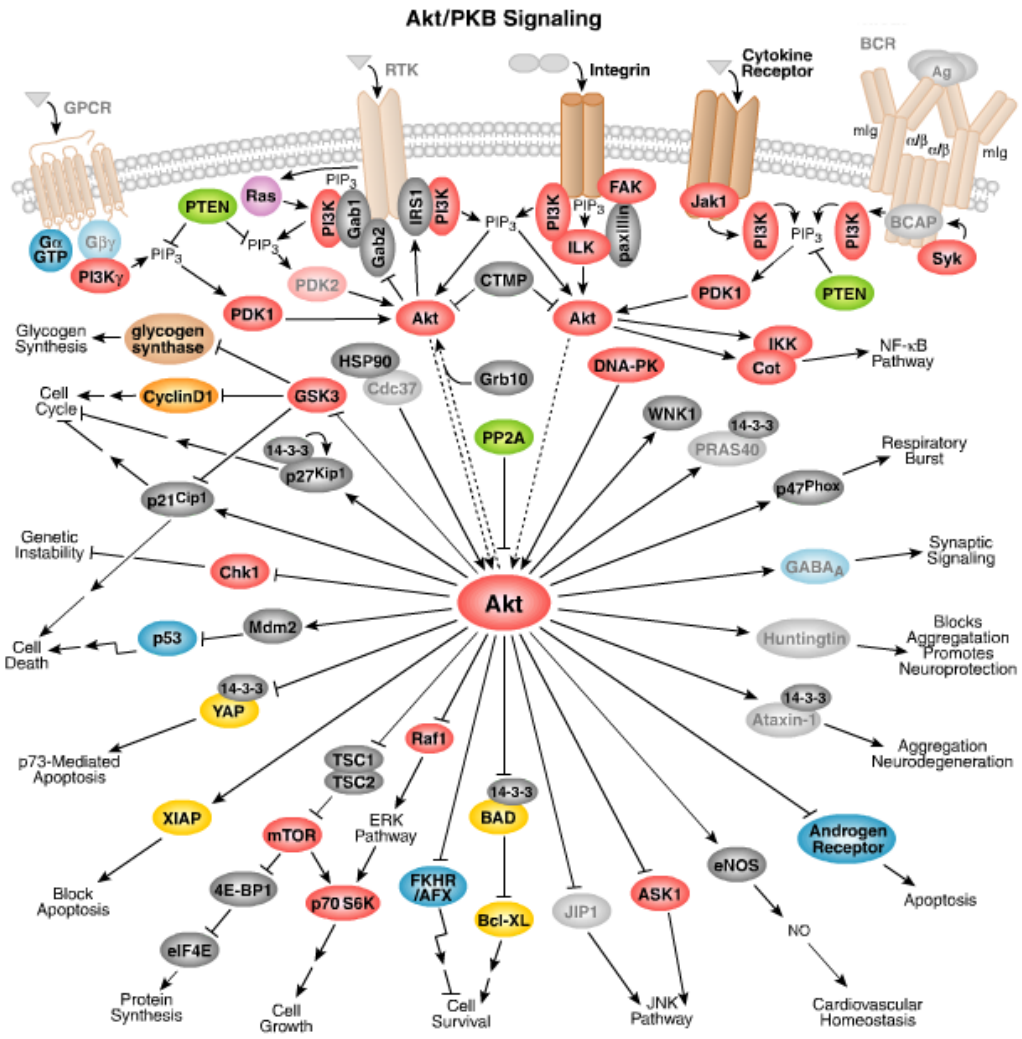

Figure 6.5.1 The many roles of mammalian AKT.
Many cell-surface receptors induce production of second messengers like PIP_3, that convey signals to the cytoplasm from the cell surface. PIP_3 signals activates the kinase PDK1, which in turn activates the kinase AKT. Proteins phosphorylated by activated AKT promote cell survival and oppose apoptosis and longevity. Aside from these biological processes, it is also important in development and growth processes such as morphogenesis. Figure taken from Cell Signaling Technology website (May 2009).

The lifespan data from the hypomorphic mutants suggests that an unknown factor may either exist or is missing in the $w^{Dahomey}$ background and it is this that enables dAkt mediated lifespan extension. For example, one sex or one background feeding more than another could result in an increase in the basal level of II signalling, and thus may respond differently to IIS disruption. As shown in chapter 5, females do consume a greater volume of food than males, and consequently, the volume consumed may vary across a greater

range. This variation of food intake may reflect the level of eggs each female produces, and thus the level of IIS flux in females may vary more than in males.

Reported in chapter 4, $w^{Dahomey}$ females compared to w^{1118} females have a greater response in lifespan and egg production to dietary restriction and varying yeast concentration. The different response to IIS disruption observed in the backgrounds may be an explanation to how $w^{Dahomey}$ females produce a larger number of eggs than w^{1118} females (**Figure 4.3.2** and **6.3.3**). Additionally, how $w^{Dahomey}$ females were more acutely responding in terms of lifespan to food dilution (greater median lifespan at 0.5x SYBrewer's food, **Figure 4.3.2**) than w^{1118} females. Thus, different responses to nutrition may also play a role in how lifespan responds to IIS disruption. Indeed, this is not the first observation that the lifespan response is dependent on the sex the mutation is measured in. *chico*^{1} homozygote females are long-lived whereas homozygote males are short-lived (Clancy *et al*., 2001), while over-expression of dFOXO extended lifespan in females but not in males (Giannakou *et al*., 2004).

This chapter has shown that the lifespan response to IIS manipulation in flies is variable and is not only dependent on the level of manipulation but also on the background and sex the manipulation occurs in. Too often conclusions are made from studies of IIS manipulation, where the response of IIS disruption was only measured in one background (Clancy *et al*., 2001; Tatar *et al*., 2001; Giannakou *et al*., 2004; Hwangbo *et al*., 2004; Broughton *et al*., 2005), when in fact, the situation may be more complicated. Indeed, it has been shown recently that crossing, Krebs cycle mutant *Indy*, into the w^{1118} background resulted in a suppression of lifespan extension (Toivonen *et al*., 2007; Helfand *et al*., 2009; Toivonen *et al*., 2009) which has previously been reported as long-lived (Rogina *et al*., 2000). Lifespan extension by *dAkt* disruption has been shown here to be possible but more work is required to explain why and how longevity is maximised only under certain circumstances.

6.5.3. *dAkt* mutants display an increased initial risk of mortality but a decreased mortality trajectory

Reduced IIS increases lifespan in invertebrates (Johnson, 1990; Kenyon *et al*., 1993; Clancy *et al*., 2001; Tatar *et al*., 2001) and mammals (Bluher *et al*., 2003; Holzenberger *et*

al., 2003). However, it is not known if this lifespan extension is the result of decreased accumulation of irreversible damage in a manner similar to reduced ambient temperature in flies (Loeb and Northrop, 1917) or a reduction in the transient risk of death in a manner similar to the effect of DR in flies (Mair *et al*., 2003). The mortality analysis suggests that experimental flies had an increased initial rate of mortality but decreased mortality trajectory rate, except in the $w^{Dahomey}$ males, where the opposite was true. This suggests that IIS reduction by *dAkt* disruption triggers mechanisms which protect against ageing rather than a short term risk of death. Furthermore, it suggests that IIS reduction is a risky strategy of lifespan extension as physiologically it may be detrimental to health, for example, diabetes in humans. The mortality data from females replicated the findings in $chico^{1}$ females reported in Tu *et al.* (2002), but contradicted the findings of Clancy *et al*. (2001), Broughton *et al*. (2005) and Giannakou *et al.* (2007), where $chico^{1}$ homozygous flies, MNC-ablated flies and flies with dFOXO over-expression show reduced initial rate of mortality but not mortality rate. This suggests that *dAkt* flies either extend lifespan by another manner from the other IIS mutants reported, or the increased initial rate of mortality is an indicator that the flies were sick.

Tissue-specific, inducible induction or suppression of single genes is now becoming increasingly feasible in biological research. Work in *C. elegans* on the stages in life in which reduced IIS functions to extend lifespan demonstrated that the later the reduction of IIS flux occurred in the life of the worms, the smaller the life-extension seen (Dillin *et al*., 2002). However, this was determined by analysing the effect of progressively delayed reduction of IIS flux on median lifespan, and both interventions that reduce risk and those that reduce permanent damage would have this effect on median lifespan. Therefore, one could apply a method whereby IIS reduction begins to occur at different stages of adulthood. This may be productive in determining if reducing signalling through the IIS pathway decreases the levels of irreversible damage or reduces the transient risk of death. Indeed, analysis of dFOXO induction at various stages in life have shown that dFOXO, and thus IIS disruption, may prevent ageing by reducing risk early in life but does so by reducing damage later in adulthood (Giannakou *et al*., 2007).

6.5.4. *dAkt* mutants and increased resistance to oxidative stress

Increased resistance to physiological stress, such as oxidative stress, is often correlated with life-extending genetic alterations (Clancy *et al*., 2001; Hwangbo *et al*., 2004; Broughton *et al*., 2005). In flies, oxidative stress is often administered by paraquat addition to fly food. However, such reports of oxidative stress resistance do not measure whether cohorts of flies alter rates of food intake, especially as the presence of paraquat has been found to reduce the meal size of flies (Ja *et al*., 2007). Thus, resistance to oxidative stress may be attributable to a reduced dose of paraquat. Such flaws may explain the variation to oxidative stress between hypomorphs in differing genetic backgrounds. All experimental females in the $w^{Dahomey}$ background subjected to paraquat treatments showed increased resistance to oxidative stress, whereas $dAkt^{+/-}$ females in w^{1118} did not display increased resistance. This may be due to differences in food intake between backgrounds. Furthermore, only two ($dAkt^{5F3/-}$ and $dAkt^{6W2/-}$) of the three female hypomorphs also displayed resistance to hyperoxia whereas resistance to paraquat was present in all. However, the conclusion drawn from analysing *chico* heterozygotes for feeding differences from their controls suggest that IIS mutants do not alter their food intake (**5.4.3**).

Finally, paraquat resistance was observable in flies that were fed RU486. *dAkt*-RNAi, *Dp110*-RNAi flies, as well as the control flies that were fed RU486 were all observed to have increased survival to paraquat. Despite this increase, induced RNAi lines still displayed better oxidative resistance than induced control lines. Interestingly, this effect was more visible in abdominal fat body S_1106 driven flies and less visible in ubiquitously *tubGS* driven flies. Indeed, oxidative stress resistance was only noticeable in dFOXO flies that were only over-expressed in the head fat body and not the gut and abdominal fat-body (Hwangbo *et al*., 2004). This indicates that either the gut and abdominal fat-body driver, when induced by RU486, provides a protective effect against oxidative stress which obscured any observation of resistance or RU486 sufficiently deterred feeding in flies which obscured any observation of resistance due to their decreased level of paraquat intake.

Chapter 7: General discussion and conclusions

7.1. Summary of findings

In this thesis, I demonstrated that it is possible to recreate the appearance of dietary restriction (DR) by food dilution in *Drosophila melanogaster*, when in fact the increased fly lifespan was a response to rescue from a harmful factor, such as excess salt in the food or water stress. However, the DR diet consisting of SYBrewer's yeast did produce a genuine lifespan extension because at increased food concentrations female egg production was also increased and the corresponding reduction of lifespan could not be rescued by addition of a water source. When tested on a range of inbred laboratory fly lines, this lifespan response was not affected suggesting DR is not a laboratory artefact resulting from years of selective breeding and that food dilution is an effective method of DR implementation in flies. Furthermore, flies on a DR diet do not compensate their reduced nutritional intake with increased feeding rate throughout their lifetime. This view is supported with studies using an optimised protocol for measuring food intake under undisturbed conditions in flies. Additionally, the dAKT/PI3K signalling pathway was demonstrated to play a role in fly lifespan, with mutants and RNAi lines displaying increased longevity. Furthermore, metabolic energy stores, stress resistance were also observed to be increased. However, the lifespan response is sensitive to the level of insulin/insulin-like signalling (IIS) modulation and the genetic background flies descend from.

7.2. Discussion and future directions

7.2.1. Technical considerations before DR implementation in flies

One of the aims of this thesis was to add to the known data in the field and further characterise the response of *D. melanogaster* to DR, with a view to better understanding the mechanisms by which it extends lifespan in this species. DR has been reported to increase the lifespan of the fruit fly in a multitude of studies by different laboratories (Van

Herrewege, 1974; Driver and Lamb, 1980; Chippindale *et al*., 1993; Chapman and Partridge, 1996; Clancy *et al*., 2002; Pletcher *et al*., 2002; Rogina *et al*., 2002; Magwere *et al*., 2004; Rogina and Helfand, 2004; Wood *et al*., 2004; Bross *et al*., 2005; Mair *et al*., 2005; Zheng *et al*., 2005; Min and Tatar, 2006b; Libert *et al*., 2007) and also in many other animals (reviewed in Weindruch and Walford, 1988). However, it is still unknown if lifespan extension under DR is achieved through common mechanisms in different species. A case for conservation of the mechanisms by which DR extends lifespan can be made from evolutionary considerations. The theory is that individuals may divert resources away from reproductive output and into somatic maintenance during times of food scarcity. By this means, they may survive periods when it would be futile to attempt reproduction, thereby successfully postponing reproduction for a later date in which times are more plentiful (Masoro and Austad, 1996). While DR effects on lifespan are widespread amongst animal species, there is no guarantee that the biological mechanisms involved are the same. Quite possibly, the effects of DR on ageing in different animal groups reflect convergent evolution, and involve different mechanisms in different taxa or at least, a combination of mechanisms that are and are not evolutionarily conserved. Thus, there is a risk that studies of DR in *D. melanogaster* may lead to the discovery of at least some mechanisms of ageing that are fly specific, and therefore, of more limited interest. Yet this potential disadvantage is counter-balanced by the great convenience of this organism as an experimental model (e.g., its 2-3 month lifespan compared to many years in rats and mice), and the possibility of relating the biology of DR to the growing understanding of the broader biology of ageing in this organism. To resolve the issue of whether DR is evolutionary conserved, it is essential that studies of DR are reported in sufficient detail for the procedures to be replicated and that any discrepancies found are not the result of technical issues or differences.

Avoiding food toxicity in food dilution

During experiments to optimize diets for lifespan studies, conditions were found in which substitution of the yeast component with yeasts from different suppliers could alter, or even eliminate the DR effect. Furthermore, because the food was both the nutrient and water supply, increased nutritional value of the food were found to increase the ratio of dissolved nutrients to water, and thus, caused the flies to suffer shortened

lifespan due to increasing food hardness and/ or water stress. The situations described may have resulted in the claim that fruit flies elevate their feeding in response to lowered nutritional intake (Carvalho *et al*., 2005), when in fact, flies decreased their feeding in response to increased toxicity in concentrated food (**Chapter 3** and **5**). One method to gauge whether flies are exposed to a detrimental factor in increasing food concentration is to measure female fecundity as a marker of health. If egg-laying increased and lifespan decreased with food supply, then it is reasonable to deduce that this response is based on increased nutrient intake. If both egg-laying and lifespan in females did not increase as nutrient level increases, then the food may be having a detrimental effect on health. A further test for food toxicity could be made using behaviour assays such as negative geotaxis (Martin and Grotewiel, 2006) on young flies. Unfortunately, not all studies display these precautions, for example, work on the effects of dietary lipids on lifespan was performed without any simultaneous measure of egg-laying or activity, thus making it impossible to know if increased food supply was in fact associated with increased nutrition, or if the short lifespans associated with elevated lipid supply were due to either a nutritional effect or toxicity of the lipid sources added (Driver and Cosopodiotis, 1979; Driver and Lamb, 1980; Driver *et al*., 1986).

Avoiding differences in mating history

All the experiments performed in this thesis studied flies in single-sex groups. However, many studies that investigate the mechanisms of DR have done so using mixed-sex groups (David *et al*., 1971; Van Herrewege, 1974; Chippindale *et al*., 1993; Bradley and Simmons, 1997; Good and Tatar, 2001; Rogina and Helfand, 2004; Wood *et al*., 2004; Bauer *et al*., 2005; Bross *et al*., 2005; Zheng *et al*., 2005; Min and Tatar, 2006a, b). In *Drosophila*, high levels of nutrition increased female egg production and lead female flies to use up their supplies of stored sperm more rapidly (Trevitt *et al*., 1988) and to re-mate more frequently than those with poorer nourishment (Harshman *et al*., 1988; Chapman and Partridge, 1996). Additionally, sexual activity has been shown to shorten the lifespan of both male and female flies (Partridge and Farquhar, 1981; Partridge and Andrews, 1985; Partridge *et al*., 1987; Fowler and Partridge, 1989; Prowse and Partridge, 1997; Wigby and Chapman, 2005). Thus, flies maintained on high-nutrient food that are allowed to mate freely throughout life will be shorter lived than those on low-nutrient food, due

to differences in sexual activity, as well as differences caused by direct nutritional effects of DR. Studies that used mixed sexes are also confounded by differences in the sex-ratio of treatments that emerge as the experiments progress, since those treatments with high mating rates will become male biased earlier in the study than those with low mating rates, as females suffer the deleterious effects of being mated at high rates (Fowler and Partridge, 1989). Thus performing DR experiments on flies in mixed-sex groups result in comparing flies subjected to both different nutritional histories and different mating histories. Thus, it is essential for any study investigating the effects of DR on fly lifespan to control mating status by using single-sex cohorts.

Furthermore, increasing the opportunity for sexual selection (through either female choice or male-male competition) over many generations have been found to genetically correlate with increased adult survival rates (Promislow *et al*., 1998). Thus, it is important that flies in stocks are maintained in sex-ratio balanced populations, something which can be affected by the presence of *Wolbachia* (Charlat *et al*., 2003). However, removal of *Wolbachia* by tetracycline-treatment from the fly lines made no difference to the measured lifespan or DR response (**Chapter 4**), although it is still important to state whether *Wolbachia* was present or not in stocks for other experiments, as evidence exists that its presence can affect lifespan and be detrimental to it (Min and Benzer, 1997).

Avoiding differences in stock maintenance

Although, not only applicable to DR experiments, it is important to control other factors that may influence the lifespan of flies and obscure any potential findings. One such factor that may play an important role in lifespan is how fly stocks are maintained in the laboratory. For experiments in this thesis, Dahomey fly stocks were maintained in overlapping generations in a large population cage, but mutant stocks were kept in bottle culture that were transferred to fresh food culture every 4 weeks. In a 4-week stock culture at 18^{o}C, adult flies are transferred to fresh bottles in which eggs are laid. Only those eggs laid within the first 48h are likely to contribute to the following generation due to competition and time constraints (Promislow and Tatar, 1998). Development from egg to adult takes about 19 days at 18^{o}C (9-10 days at 25^{o}C) (Ashburner, 1989). Thus, by the time of a new round of stock transfers, flies transferred, are up to a maximum of 10 days of age, all eggs laid by these adults before transfer make no contribution towards lifetime

reproductive success. Then, within 48 h, all flies experience a narrow window of potential reproductive opportunity. As a consequence, genes for adult fitness traits expressed after 10 days of age are not directly exposed to selection. This truncation of adult period in bottles has been claimed to constitute a *de facto* mutation accumulation experiment, allowing late-acting deleterious mutations to increase in frequency in the base stock in the absence of selection (Promislow and Tatar, 1998). Indeed, it has been shown that such laboratory practices may contribute to a decrease in adult survival (Sgro and Partridge, 2000) either through inbreeding or early reproduction (Luckinbill *et al*., 1984; Rose, 1984; Sgro and Partridge, 1999). Despite the possible effects on lifespan as a result of differences in maintenance, the DR responses between different wild-type inbred stocks were not affected (**Chapter 4**), which suggests that the DR response is unlikely to have evolved in the laboratory, and that any possible late-acting mutations accumulated in other genetic backgrounds does not block the effect of DR.

Other technical issues to consider

Initially reported in rotifers, the longevity of offspring has been found to be inversely proportional to the age of its parents (Lansing, 1947). Known as the Lansing effect, it has since been discovered in *D. melanogaster*, where old mothers have also been found to produce short-lived offspring (Priest *et al*., 2002), and more recently, evidence suggest that this effect may be inherited genetically (Spencer and Promislow, 2005; Yilmaz *et al*., 2008). Thus, all flies generated for experiments in this thesis were generated from age-matched parents to reduce this risk.

The density of flies in bottles/ vials has been recorded to be inversely proportional to their lifespan (Pearl, 1928) and fecundity (Pearl *et al*., 1926). In addition, flight activity also reduces the lifespan of house flies (Yan and Sohal, 2000) and *Drosophila* (Magwere *et al*., 2006). Thus, studies performed with bottles generally produce shorter lifespan (Mair *et al*., 2003) and must be taken into consideration when comparing studies with vials. The difference in longevity between individually and group-housed flies at different food levels remains untested, although feeding experiments suggest flies increase their food intake when housed in larger groups (**Chapter 5**).

Conclusion

Despite many years of work, the mechanisms that underlie the effect of DR on lifespan remain unknown. Although historically much of the work has been performed with rodents, large-scale lifespan experiments under many conditions and genetic analysis are better suited to shorter lived, and more easily housed, model organisms such as *Drosophila*. However, if work in the fruit fly is to be of any relevance to the study of ageing in higher organisms, it is important that techniques established here which eliminate some of the confounding effects of non-ageing-related causes of death, such as food toxicity, are used. Only then can the mechanistic relationship between diet and death be established, providing modes of action to be tested in the longer lived models. Although the results in this thesis do not eliminate all of the possible confounding effects, it provides a solid technical foundation for future DR experiments in the fruit fly.

7.2.2. Dietary restriction by reduction of nutritional intake

The lifespan response of DR is widely believed to be the result of caloric restriction (Weindruch and Walford, 1988; Bordone and Guarente, 2005; Masoro, 2005, 2006), however recent findings have challenged this view suggesting that specific nutrient restriction (proteins and amino acids) rather than energy restriction is responsible (Zimmerman *et al*., 2003; Mair *et al*., 2005; Piper *et al*., 2005). One problem with this current view of DR is that there is no accepted level of nutrient intake to use as a reference, which results in two major considerations: what is the nutrient restriction relative to? And is it yeast restriction that prolongs longevity or too much yeast consumption that shortens lifespan? (Raubenheimer *et al*., 2005; Piper and Partridge, 2007; Simpson and Raubenheimer, 2007). Despite discounting the possibility of the latter in chapter 3, the DR dilution method does not involve direct quantification of the volume of food intake, thus DR is assumed to have taken place even though little work has been done to test for any compensatory feeding or changes in the ratio of nutrients in the diet during feeding.

Insects often experience significant variability in food quality throughout their lifetime, both because the general nutritional quality of food varies considerably in space and time (Scriber and Slansky, 1981) and because the nutritional needs of insects vary with the

changing requirements of growth, development and reproduction (Raubenheimer and Simpson, 1997; Warbrick-Smith *et al*., 2006). Thus, the idea of insects being able to compensate and maintain an optimal or near-optimal nutritional intake is not far-fetched. Three stages of the feeding cycle in insects have been especially studied for adaptive responses: (1) *dietary selection*, where certain insects have been shown to select, from available foods, a mixture that supports growth and development better than any single food alone (Messina, 1982; Waldbauer and Friedman, 1991; Simmonds *et al*., 1992; Lee *et al*., 2002; Lee *et al*., 2006; Raubenheimer *et al*., 2007) (2) *food consumption*, where certain insects have been shown to alter either the meal size or the inter-meal interval to compensate for reduced nutrition (Gelperin, 1971; Barton Browne, 1975; Bailey and Mukerji, 1976; Rachman, 1980; Simpson *et al*., 1989; Raubenheimer, 1992; Yang and Joern, 1994a) and (3) *post-ingestive food utilisation*, where certain insects manipulate their gut size as a way to mitigate the difference between their nutritional and metabolic requirements, and variable supplies of energy and nutrients from the food (Bailey and Mukerji, 1976; Jindra and Sehnal, 1989; Zanotto *et al*., 1993; Yang and Joern, 1994b; Zanotto *et al*., 1996). Such mechanisms have also been reported to be at work in *Drosophila* and thus can be a major concern for experiments involving DR.

Choice experiments have revealed that adults flies are able to dietary select for an optimal protein to carbohydrate ratio that maximises lifetime egg production but at a sacrifice to longevity (Lee *et al*., 2008). While larvae are able to select for a protein-rich food source (soybean) over a carbohydrate-rich medium (corn-based) (Ryuda *et al*., 2008). This provides evidence for not only that *D. melanogaster* are capable of dietary self-selection (if choices are available) but also for the role excess protein has in life-shortening. Interestingly, in choice-less experiments, where protein: carbohydrate is fixed, flies are unable to compensate for any lowered protein to carbohydrate ratios because they are limited by the volumetric constraints of total intake (Lee *et al*., 2008). Experiments measuring food consumption have shown that fruit flies can compensate their feeding when subjected to sucrose concentrations below 50mM (Edgecomb *et al*., 1994), however, standard DR food contains a sucrose concentration that are above this threshold, and thus are not expected to trigger this response. Indeed, quantification of food intake by proboscis-extension calibrated by short-term dye measurements has suggested that partial or total nutritional compensation does not occur (**Chapter 5**). Thus,

flies subjected to lowered nutritional content in the food (DR) do indeed receive lowered nutrition. However, findings that suggest DR flies have increased gut volume compared to fully fed flies (**Chapter 5**) and therefore restricted flies may increase food absorption (post-ingestive compensation) by this method. Such digestive adaptations may indicate restricted flies extract more relative protein and carbohydrate from the diet during absorption than fully fed females, and so, even if the nutrition acquisition of restricted flies were controlled, it may not reflect the level of nutrient assimilated. One recent DR study has measured the assimilation and allocation of nutrient by feeding dietary yeast cultured in medium containing the stable isotopes ^{13}C and ^{15}N to flies (O'Brien *et al*., 2008). Females on a full diet were found to acquire and allocate more dietary carbon, nitrogen and essential amino acids to both eggs and somatic tissues than females on a DR diet (O'Brien *et al*., 2008). Thus, the longer lifespan of flies on a DR diet relative to those on a full diet is related to the lowered nutrition that DR flies receive. Furthermore, this lifespan difference cannot be explained solely by greater total somatic investment, and high somatic investment does not ensure longevity, countering the idea that DR extends lifespan by a resource trade-off between somatic repair and reproduction (Kirkwood, 2005).

A key question that arises is what are the life-shortening consequences of ingesting excess nutrients? One explanation is that the shortened lifespan is the result of the greater damage associated with increased reproduction, which is a consequence of a fully fed diet (Leroi, 2001; Partridge *et al*., 2005). Indeed, allocation of resources to somatic tissue relative to investment to eggs was found to be greatest in females on a restricted diet, even though fully fed females had greater total allocations to somatic tissue (O'Brien *et al*., 2008). This suggests that the greater reproduction in fully fed females and the damage that came with it, was off-setting any benefits of increased resources for somatic allocation. Another explanation is that nutrient restriction either decreases mitochondrial generation of reactive oxygen species (ROS) or increases the repair to oxidative damage (Barja, 2002), molecular factors which may cause ageing. One study found that protein restricted rats had a more efficient respiratory chain which prevented ROS production and free radical leak than in fully fed rats (Sanz *et al*., 2004). However, recent studies have also suggested that DR does not prevent ROS-induced damage accumulation in *D. melanogaster* (Edman *et al*., 2009), while work in *C. elegans* have shown that increased

enzymes which counter oxidative stress have little effect on lifespan (Doonan *et al*., 2008; reviewed in Gems and Doonan, 2009). A final explanation is that nutrient restriction increases lifespan by affecting the IIS pathway (Clancy *et al*., 2002; Giannakou *et al*., 2008; Min *et al*., 2008) and/ or amino acid signalling pathways (e.g., target of rapamycin, TOR) (Kapahi *et al*., 2004).

7.2.3 Dietary restriction and insulin/ insulin-like signalling

Signalling pathways mediate the necessary flexibility of any organism's metabolic network to changeable nutrition. The IIS and TOR signalling pathways have been identified in flies to couple growth to nutrition as well as playing a role in the control of adult lifespan (Clancy *et al*., 2001; Tatar *et al*., 2001; Giannakou *et al*., 2004; Hwangbo *et al*., 2004; Kapahi *et al*., 2004; Broughton *et al*., 2005). One recent focus in ageing research has been to determine whether lifespan extension by DR involves these mutations in IIS and TOR because these mutations have been found to affect the nutrient responses of metabolism, growth and reproduction, such as in the case of the *dAkt* mutants (**Chapter 6**) (Partridge *et al*., 2005b; Tatar, 2007). If DR is dependent on these signalling pathways, the relationship between longevity and nutrition should be different between the mutant and its control over a range of food concentrations, because the IIS mutant flies are already to some extent subjected to a form of DR, whereas the control is not. Thus, mutants will be more prone to starvation than controls, but more resistant to increased food concentrations. This would be observable as a shift to the right of the "DR tent", a description of how lifespan behaves over a range of food dilution (**Figure 7.1**). Conversely, if food dilution induces the same degree of longevity extension at similar food concentrations in a mutant and its control, then DR must work independently of the gene's product.

The interaction between DR and the lifespan-extension conferred by the IIS mutation has been reported in *chico*^{1} flies (the IIS receptor substrate) (Clancy *et al*., 2002) and in flies over-expressing dFOXO (a transcription factor antagonised by IIS) in the gut and abdominal fat body (Giannakou *et al*., 2008), as has the interaction between DR and reduced TOR-pathway activity in flies over-expressing dTSC2 (an antagonist of dTOR) (Kapahi *et al*., 2004). These studies show that the peak lifespan of IIS and TOR disrupted

flies occurs at a higher food concentration than the peak lifespan of the control flies, whereas the same flies have shortened lifespan at lower food concentrations (more sensitive to starvation). This supports the idea that flies are already partially dietarily restricted by their genotype because the effect of DR is mediated by these nutrient sensing pathways. Another explanation is that IIS or TOR disruption results in lowered food intake behaviour and flies are thus subjected to a different form of DR; however feeding measurements of *chico*^{1} heterozygotes show no differences in food intake (**Chapter 5**).

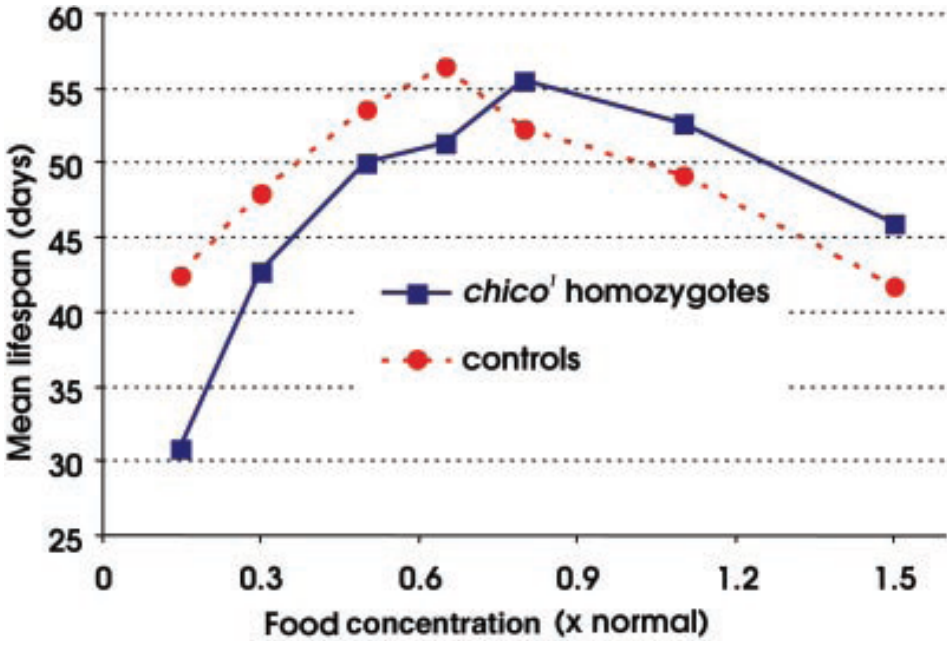

Figure 7.1 Interaction between DR by food dilution and reduced IIS in the determination of mean lifespan.
As food concentration increases, lifespan increases from starvation to a peak, after which increased concentration results in a reduction of lifespan. *chico*^{1} homozygotes are partially restricted as a result of their mutation, which shifted the DR tent/parabola to the right of controls, and also resulted in the greater susceptibility to starvation than controls at lowered food concentration. This provides evidence that DR and IIS operate through overlapping mechanisms. Figure taken from Clancy *et al.* (2002).

On the other hand, another study has showed that *dFOXO* over-expression in the head fat-body resulted in increased lifespan at high levels of food concentration compared to controls, but unlike Giannakou *et al.* (2008), did not increase sensitivity to starvation (Min *et al*., 2008). Min *et al.* (2008) also found that *dFOXO* over-expression in the gut and abdominal fat-body consistently increased lifespan at all food concentrations over controls rather than shifting to the right (an indicator that dFOXO over-expression resulted in partial DR, **Figure 7.2**), which led them to conclude that DR is independent of

IIS. Furthermore, disruption to IIS and TOR signalling does not completely abolish lifespan variation in response to altered nutrition (Clancy *et al*., 2002; Kapahi *et al*., 2004; Giannakou *et al*., 2008), which would be expected if the intervention were solely responsible for mediating the effects of DR. This remaining lifespan variation could be mediated either by residual signalling activity in the mutated pathway (none of the interventions completely abolishes signalling through its respective pathway) or indeed due to the existence of alternative, parallel, nutrient sensing pathways that also affect lifespan variation.

Experiments with *dFOXO* null mutants were also found to respond to DR with increased lifespan as food was diluted (Giannakou *et al*., 2008; Min *et al*., 2008). This suggests that in the absence of active IIS, and more specifically in the absence of the FOXO transcription factor target of IIS, other pathways are able to mediate the response to DR. However, further dilution of food concentration resulted in a decrease in lifespan as a result of starvation of *dFOXO* nulls (Giannakou *et al*., 2008), whereas under a different dietary yeast, no starvation response was once again observed (Min *et al*., 2008).

This reveals a more complex interaction of the mutation with diet and can explain how different laboratories using a single, but different, diet could find opposing effects of a mutation on lifespan. It also shows how a range of diets of different compositions could lead to varying interpretations of how the two interact mechanistically to extend life. Indeed, over-expression of *dFOXO* produced a qualitatively different response of lifespan to food concentration when DR was either implemented by reducing yeast alone, or by reducing sugar and yeast together (Giannakou *et al*., 2008). Thus, it is not clear whether all mutations involved in nutrient and endocrine signalling operate in the same pathway or that they operate to modify the response of lifespan to dietary composition that varies across different DR methods.

Reduction in food intake is likely to influence a host of nutrient sensing and metabolism-related pathways and as such it seems counter-intuitive that the physiological changes induced by DR should affect only one factor (e.g. IIS pathway). Thus, the same method of DR implementation in the same organism may not result in the same outcome if the suspected factors are different. For example, in mice, reduction in food intake from *ad libitum* to 70% starting at approximately 2 months of age produced

further significant extension of average and maximal longevity in Ames dwarf (*Prop1df*) mice (Bartke *et al*., 2001). Whereas, an identical dietary regimen had no effect on longevity of GHRKO (Growth Hormone Receptor Knockout) males and produced only a modest increase in maximal longevity without affecting the median or the average life span in GHRKO females (Bonkowski *et al*., 2006). This result was unexpected because both sets of mice were characterised by extremely low levels of IGF-1, and thus were assumed to interact closely (Bartke, 2008).

Various methods are used to implement DR within and across organisms, and it is possible that different DR methods may not at all be acting through the same pathways. For example in *C. elegans*, studies have often used different nutritional or genetic intervention methods for implementing DR and assumed that various DR methods interact or overlap between them. However, *eat-2* mutants, which are thought to be restricted because of their reduced feeding, had further increased lifespan when subjected to bacterial food restriction (Hansen *et al*., 2007), implying that *eat-2* DR and bacterial dilution DR in *C. elegans* might be acting through different mechanisms. Furthermore, as described here in *dFOXO* studies, the different dietary yeasts used may have a profound effect on the way a result is interpreted (Giannakou *et al*., 2008; Min *et al*., 2008).

In order to informatively interpret data from such studies the diet must be fully maximised for both cohorts. For example in **Figure 7.2A**, as food intake is reduced from *ad libitum* levels, lifespan gradually increases until a food level that maximizes longevity is reached, past which further reduction in food intake begins to shorten lifespan as animals enter starvation. In Min *et al.* (2008) (**Figure 7.2B**) the yeast diet used clearly does not result in a traditional parabolic DR response as no starvation response to lowering food concentration is observed in either experimental or control lines. The food concentration range measured in this experiment may be between food concentrations *C* and *D* in **Figure 7.2A**, and thus, no response to starvation was measured rather than no response to starvation exists. This may also account for the general lower mean lifespan of the flies when compared with data from Giannakou *et al.* (2008) (**Figure 7.2C**). Further dilution of this dietary yeast is therefore required to determine whether the lifespan of dFOXO-induced flies are shifted upwards or towards the right on the graph.

If lifespan extension due to DR is dependent upon a single factor, removal of that factor will completely abolish the DR effect. Rather than a parabolic response of longevity to DR there will be no interaction between food intake and lifespan when the DR factor is removed. Longevity will be the same at all food concentrations and plotting average lifespan against food concentration would produce a horizontal line across the DR range. At the present time, it is impossible to arrive at any definitive conclusions on whether diet and lifespan in *D. melanogaster* are connected via the nutrient sensing pathways as no experiments have been performed to test genotype by diet interactions using full and precise nutritional manipulations.

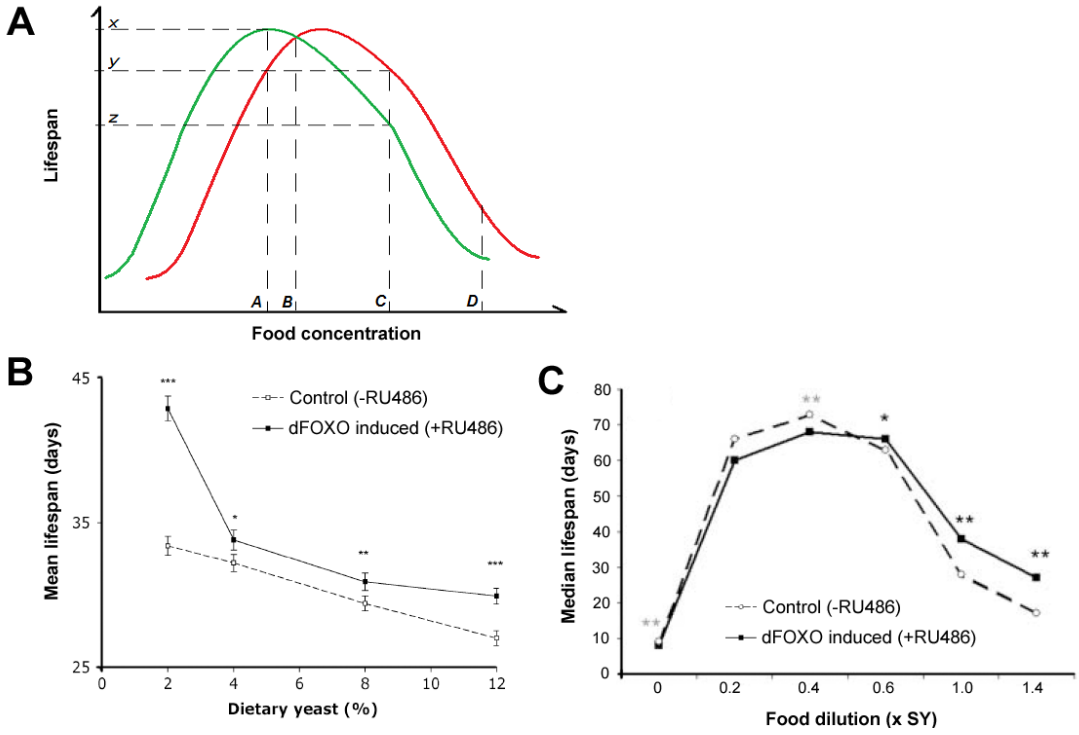

Figure 7.2 Measuring lifespan without a full range of food concentrations can lead to misinterpretation of DR.

(**A**) The lifespan response of WT animals to increasing food concentration follows a parabolic shape (green). Lifespan increases from starvation to a peak before decreasing as food concentration continues to increase. If lifespan at two food concentrations were measured (*A* and *C*) then a "DR response" is observed in WT lifespan (*X* and *Z*, respectively). If a genetic mutation alters the response of an animal to DR, the position of this curve on the x-axis can be shifted right (red line) (Clancy *et al*., 2002). In this situation, if the mutant lifespan at the two food concentrations were measured again then no difference in lifespan (only *Y*) is perceived. Testing a full range of food concentrations rather than just the two would prevent this misinterpretation. Furthermore, measuring lifespan only at food concentration *B* would result in the conclusion that a genetic mutation does not affect lifespan. (**B**) DR in adult *Drosophila* when dFOXO is over-expressed in visceral fat body by induction with RU486 via P[Switch]$S_1$106 relative to the same genotype without RU486 (vehicle only control). Mean lifespan with standard error. Asterisks indicate yeast concentrations where survival differed significantly by log-rank test (**P* < 0.05; ***P* < 0.01; ****P* < 0.0001). Panel (**B**) is taken from Min *et al.* (2008). (**C**) Median lifespans of $S_1$106/UAS-dFOXO female flies across a food dilution series on +/– RU486 SY food. (**P* < 0.05 and ***P* < 0.0001). Panel (**C**) is taken from Giannakou *et al.* (2008).

Performing lifespan assays with limited food concentrations carry a risk of misinterpretation in DR studies. However, this risk is also apparent to assays that test whether genes may or may not play a role in lifespan determination. For example in **Figure 7.2A**, if mutants for a candidate gene (red line) were tested with its control (green line) for lifespan differences at only food concentration *B*, no difference in lifespan would be detected, an incorrect conclusion. Lifespan studies investigating the role of *dAkt* disruption in fly lifespan was performed at one food concentration (**Chapter 6**). If the yeast concentration that flies were exposed to was at the hypothetical food concentration *B*, then it may explain why *dAkt* hypomorphs had such varied lifespan data, with both lifespan extension and shortening recorded. Thus, it would be of great interest to test the various *dAkt* hypermorph mutants on a full range of nutrient concentrations, which is not limited to solely manipulating yeast concentration but a fully defined synthetic media with relevant alterations to protein, lipid, vitamin and minerals. This would be a truly epic study that would clarify the role of the IIS pathway in lifespan and in DR.

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Abbreviations

4E-BP – Eukaryotic initiation factor 4 Binding Protein

AL – *Ad Libitum*

CAFE – CApillary Feeder

clk – clock

CLS – Chronological Life Span

CT – Controlled Temperature

DAF – DAuer Formation

DILP – *Drosophila* insulin-like peptide

DNA – DeoxyriboNucleic Acid

DR – Dietary Restriction

EDTA - EthyleneDiamineTetraacetic Acid

EOD – Every Other Day

ERC – Extra chromosomal RNA Circle

FOXO – FOrkhead boX subgroup O

GHRKO – Growth Hormone Receptor KnockOut

GLM – Generalised Linear Model

GSK-3 – Glycogen Synthase Kinase 3

HCl – HydroChloric acid

IF – Intermittent Feeding or Intermittent Fasting

IGF – Insulin-like Growth Factor

IIS – Insulin/ Insulin-like Signalling

IRS – Insulin Receptor Substrate

KAc – Potassium Acetate

LDF – Limited Daily Feeding

LiCl – Lithium Chloride

LMEM – Linear Mixed Effect Model

MNC – Median Neurosecretory Cell

MRDT – Mortality Rate Doubling Time

NF – Number of Flies

NTP – Nucleoside TriPhosphate

NV – Number of Vials

PBS – Phosphate Buffered Saline

PCR – Polymerase Chain Reaction

PDK – Pyruvate Dehydrogenase Kinase

PH – Pleckstrin Homology

PI3K – PhosphoInositide 3-OH Kinase

PIP_2 – Phosphatidylinositol (3,4)-bisphosphate

PIP_3 – Phosphatidylinositol (3,4,5)-triphosphate

PKB – Protein Kinase B

PTEN – Phosphatase and TENsin homolog

Q – ubiQuinone

Rheb – Ras homolog enriched in brain

RNAi – RiboNucleic Acid interference

ROS – Reactive Oxygen Species

RT – Room Temperature

RT-PCR – quantitative Reverse Transcription-Polymerase Chain Reaction

S.D. – Standard Deviation

SGK – Serum and Glucocorticoid-inducible Kinase

SDS-PAGE – Sodium Dodecyl Sulfate PolyAcrylamide Gel Electrophoresis

SIR2 – Silent mating type Information Regulator-2

SIRT or SIRTUIN – SIR2 mammalian homologue

SMP – Skimmed Milk Powder

SOD – SuperOxide Dismutase

SY – Sugar/ Yeast medium

TBS-T – Tris-Buffered Saline with Tween 20

TOR – Target Of Rapamycin

Tris – Tris(hydroxymethyl)aminomethane

TSC – Tuberous Sclerosis Complex

UAS – Upstream Activation Sequence

w – *white*

wt – wild-type

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Appendix 1: Bass *et al*. (2007)

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Optimization of Dietary Restriction Protocols in *Drosophila*

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Dietary restriction (DR) extends life span in many organisms, through unknown mechanisms that may or may not be evolutionarily conserved. Because different laboratories use different diets and techniques for implementing DR, the outcomes may not be strictly comparable. This complicates intra- and interspecific comparisons of the mechanisms of DR and is therefore central to the use of model organisms to research this topic. *Drosophila melanogaster* is an important model for the study of DR, but the nutritional content of its diet is typically poorly defined. We have compared fly diets composed of different yeasts for their effect on life span and fecundity. We found that only one diet was appropriate for DR experiments, indicating that much of the published work on fly ''DR'' may have included adverse effects of food composition. We propose procedures to ensure that diets are suitable for the study of DR in *Drosophila*.

DIETARY restriction (DR) refers to a moderate reduction of food intake that leads to extension of life span beyond that of normal, healthy individuals. This intervention has principally been studied in rodents, but it also extends the life span of a wide range of organisms including the fruit fly, *Drosophila melanogaster* (1–7). Although extension of life span in response to DR is taxonomically widespread, it is unknown whether evolutionarily conserved mechanisms are at work or, instead, whether this is a case of evolutionary convergence (8). This issue is important, because upon its resolution depends the utility of the powerful invertebrate model organisms for understanding the mechanisms of the response to DR in mammals.

Considerable attention has been paid to the dietary components that are important for extension of life span by DR in rodents, where reduction of whole food intake can increase life span by approximately 40% (3). These studies have shown that altering the ratio of nutritional components, by reducing lipids, minerals, or vitamins in the diet, had no effect on rat life span, although reduction of the protein quantity or quality effected a relatively small increase (9–13). More recent work has shown that specific reduction of tryptophan (14) or methionine (15–17) can extend rodent life span to a similar magnitude as whole-food DR. On the one hand, these interventions with specific nutrients may reveal useful information about the mechanisms of whole-food DR; on the other hand, each intervention could operate through different molecular pathways to extend life span, thus revealing little or nothing about the mechanisms of whole-food DR (18). Similar debate exists over the potentially different mechanisms by which yeast replicative life span is increased when glucose is reduced from 2% to 0.05% (19) or from 2% to 0.5% (20,21). In *Caenorhabditis elegans*, several possible modes of life-span extension by food reduction exist as life span can be extended by dilution of the bacterial food source (22), complete removal of the bacterial food source (23,24), altering the strain of bacterium used in the worms' diet (25,26), or using synthetic axenic media (27,28). To establish the mechanisms at work for any particular method of DR in any model organism, precise specification and, preferably, standardization of DR methods is desirable as a basis for intra- and interspecific comparisons.

DR is usually imposed in *Drosophila* by dilution of an agar-gelled food medium, which is always present in excess (29). In general, as food is diluted from a high concentration, life span increases to a peak at intermediate nutrient levels through DR, and then falls with further food dilution through starvation. It is generally assumed that the increase in life span with DR is a response to reduced nutrients. However, logically, it could just as well be a response to relief from a nonnutritional, toxic effect of the food (30). This is not an easy issue to address empirically, but some evidence can be drawn from parallel effects of diet on reproductive output, which can provide an independent indication of the effect of the diet on the organism's nutritional status. In a manner similar to that for DR in worms and mice (22,31), a decrease in life span in response to increased nutrition should be accompanied by increased daily and lifetime fecundity (5,6). In contrast, increase in the concentration of a toxin would be expected to cause life span to decrease in parallel with a reduction or no increase in fecundity.

DR in *Drosophila* usually involves reduction of the yeast and sugar components of the diet (29), and yeast appears to account for the majority of the DR effect on life span (5,32). However, different laboratories use different sources of yeast and different concentrations of sugar, yeast, and agar for DR (5,6,33,34). Despite these differences, few laboratories have tested their diets to ensure that the effect of DR

Table 1. Recipe Used to Make Food

Media	Components	Supplier	Name
100 g	Yeast*	Baker's (B.T.P. Drewitt, London, U.K.)	SYBaker
		Brewer's (MP Biomedicals, Solon, OH)	SYBrewer
		Torula (Borregaard, Sarpsborg, Norway)	SYTorula
		Bacto Yeast extract (BD Diagnostics, Sparks, MD)	SYExtract & CSYExtract
50 g	Sucrose	(Tate & Lyle Sugars, London, U.K.)	
50 g	Cornmeal†	(B.T.P. Drewitt, London, U.K.)	
10 g	Agar	(Sigma, Dorset, U.K.)	
3 mL	Propionic acid	(Sigma, Dorset, U.K.)	
30 mL	Nipagin M‡	(Clariant UK Ltd, Pontypridd, U.K.)	
1000 mL	Made to final volume with distilled water		

Notes: The values in this table describe the arbitrary reference condition (1.0) used in dietary restriction (DR) experiments and for rearing flies. Where indicated in the text, the yeast, sugar, and agar concentrations were varied.

*For yeast comparison experiments, the yeast concentration alone was varied from 10 g/L (0.1) to 200 g/L (2.0).

†Cornmeal (organic polenta) was used for the CSYExtract medium only.

‡Solution of 100 g/L methyl 4-hydroxybenzoate in 95% ethanol.

on life span in their experiments is a specific response to nutrition as evidenced by reduced reproductive output. To gauge the importance of these differences, and to establish a validated DR diet that should be reproducible between laboratories, we assembled a range of yeast-based diets and directly compared the life span and fecundity of flies in response to DR on each food type. Of the diets that we investigated, only one showed effects on survival and fecundity that is suitable for DR studies in *Drosophila*.

Methods

Fly Stocks, Maintenance, and Handling Procedures

All experiments were performed with the wild type, outbred, laboratory strain Dahomey. The population is maintained in large population cages with overlapping generations on a 12-hour light/dark cycle at 25°C and 65% humidity.

Media

Rearing of flies and experiments were performed on standard sugar/yeast (SY) food (35). The arbitrary standard condition (1.0) is described in Table 1. In all cases, the food was prepared by adding the agar to water and bringing to a boil on a gas hob. At this point, the appropriate amounts of sugar and yeast (and cornmeal where indicated) were added with continuous stirring until the food was completely mixed. The food was then removed from the heat and allowed to cool to 65°C. At this point, preservatives were mixed in, and the food was dispensed. For the sugar range experiments, baker's yeast (Table 1) was used. Media for the comparison of dietary yeasts were based on that in Table 1, with only the yeast component varied. For the water add-back experiment, a 1% agar solution was made (containing preservatives as for the SY media) and poured into individual 200-μL pipette tips. These tips were trimmed to a length that brought the agar solution close to the level of the food surface after being inserted into the food. A pipette tip filled with cotton wool, to prevent flies from crawling into the pipette tip and becoming trapped, was added to the control treatment.

Life Span and Fecundity Assays

For life-span experiments, larvae were reared at standard density in 200-mL glass bottles containing 70 mL of 1.0 SY food (36). Flies emerged over 24 hours, were tipped into fresh bottles, and were allowed 48 hours to mate. Females were then separated from males under light CO_2 anesthesia and randomly allocated to different food treatments at a density of 10 females per vial. Flies were transferred to fresh vials, and deaths were scored at least every 2 days. The yeast comparison experiment was performed in two batches, the first containing SYBaker's, SYBrewer's, and SYTorula, and the second containing SYBaker's, SYBrewer's, SYExtract, and CSYExtract. Due to the similarity between the two trials of SYBaker's and SYBrewer's (Supplementary Figure 1 and Supplementary Table 1), the data were combined. For each condition in each experiment, 100 flies were used.

For fecundity measurements, the same experimental flies as those used for life spans were kept in the same glass vials for between 18 and 24 hours; they were then transferred to fresh food. The eggs in the vacated vials were counted manually under a microscope. For the sugar concentration experiment, egg counts were performed on days 3, 7, 10, 14, and 21 of treatment. For the first yeast comparison experiment (SYBaker's, SYBrewer's, and SYTorula), eggs were counted on days 5, 9, 12, 16, 19, 23, 26, 30; for the second experiment (SYBaker's, SYBrewer's, SYExtract and CSYExtract), eggs were counted on days 4, 8, 11, 15, 18, 22, 25, and 29. Eggs were counted on days 3, 6, 10, 13, 17, 26, 31, and 38 for the water add-back experiment and on days 4, 11, 18, 25, 32, 46, and 60 for the agar concentration range experiment. As an index of lifetime fecundity, the sum of eggs laid during 24 hours on the days of counting by an average female was calculated. These sampling points cover the period of heaviest laying, and are therefore indicative of relative lifetime fecundity (6).

Data Analyses

Comparison of survivorship data was performed using the log-rank test implemented in Excel. Values of p from comparisons of fecundity data refer to the nonparametric Wilcoxon rank sum test performed in R, v2.2.1 (37). For the

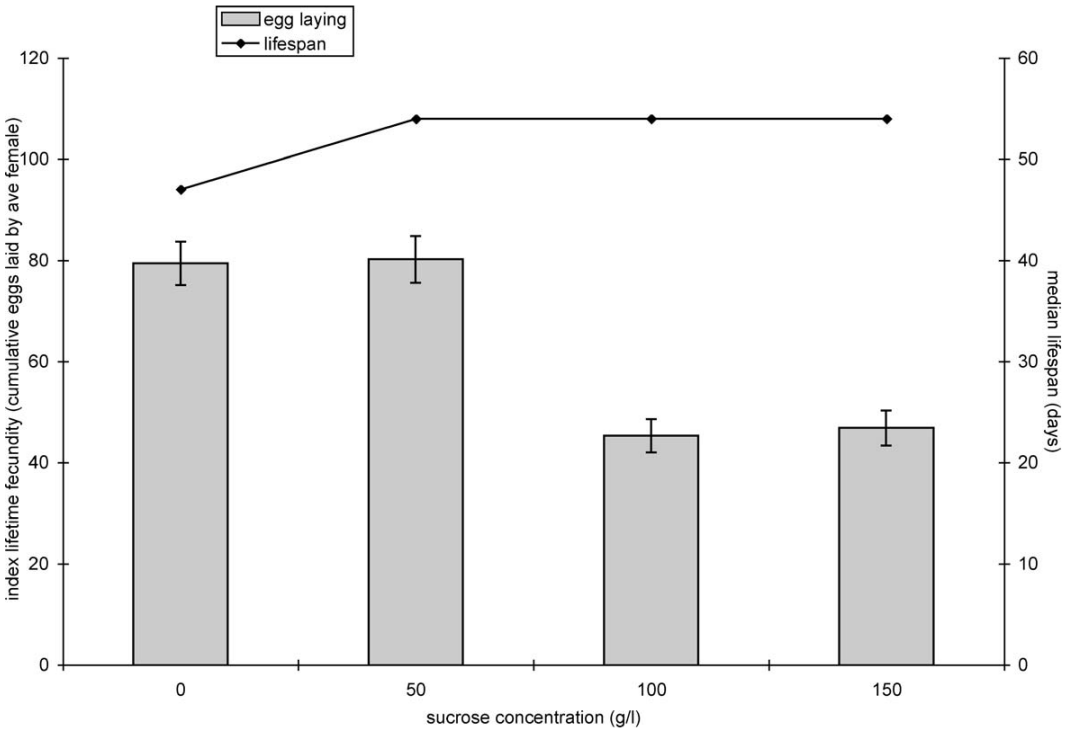

Figure 1. Effect of dietary sucrose concentration on life span and fecundity of mated *Drosophila* females. Increasing concentrations of sucrose were added to a standard food background of 1.5 SYBaker's (Table 1). Over the range of sucrose tested, very little change in life span was observed, whereas a significant decrease in fecundity was observed between 50 g/L and 100 g/L sucrose. *Gray bars:* index of lifetime fecundity (sum of the eggs laid by an average female on the days counted) ± standard error of the mean; *connected black points:* median life span. Representative data from one of two experiments are shown.

more complex comparisons of fecundity data illustrated in Figure 2, the nlme package in R was used (38), specifying a mixed model with yeast type, yeast concentration, and the quadratic function of concentration as fixed terms. Replicate vials were included as a random variable to compensate for multiple females per vial. To deal with the observed increasing variance with increasing fitted values (heteroscedasticity), we modeled the variance as a power function of the fitted values (such weighting of the variance structure improved the fit of the model, although it did not change the results). All factors and interactions were significant. Modeled versus actual data are shown in Supplementary Figure 2.

Nutritional Analysis of Yeast

Chemical analysis of a sample of baker's yeast was performed by Leatherhead Food International (Somerset, U.K.).

Results

High Levels of Dietary Sucrose Adversely Affect Fecundity With Little Effect on Life Span

Although it has been shown that the yeast component of an SY diet is critical for the response to DR in *Drosophila*, sucrose could also produce life-shortening effects similar to those of yeast if raised to sufficiently high concentrations [i.e., higher than those used previously (32)]. To test this, we looked at the effect of varying the sucrose concentration in the diet while keeping all other ingredients at a fixed level.

Interestingly, there was no requirement for dietary sucrose for maximum fecundity and, surprisingly, addition of sucrose at $\geq$100 g/L caused a decrease in female fecundity ($p < .00002$, Wilcoxon rank sum test), indicating that it had a detrimental effect on fly physiology and/or behavior. To ensure that nutrition, and therefore DR, is the key determinant of life span, fecundity should increase for increases in nutrition that cause life span to decrease. These data, therefore, show that sucrose concentrations $>$ 50 g/L are not appropriate for DR studies. For optimum longevity, the flies required the level of dietary sucrose to be at least 50 g/L in an SY diet. This effect of sucrose is shown in Figure 1 as a small, but significant, increase in median life span when sucrose was added to a yeast-only diet (50 g/L vs 0 g/L; $p < .00001$, log-rank test). Raising the sucrose concentration further to 150 g/L caused no decrease in median life span in this experiment, but it has done so in other experiments that we have performed [data not shown and (32)]. As a result, further experiments reported herein used a fixed sucrose concentration of 50 g/L as this was neither detrimental to life span nor inhibitory to egg laying.

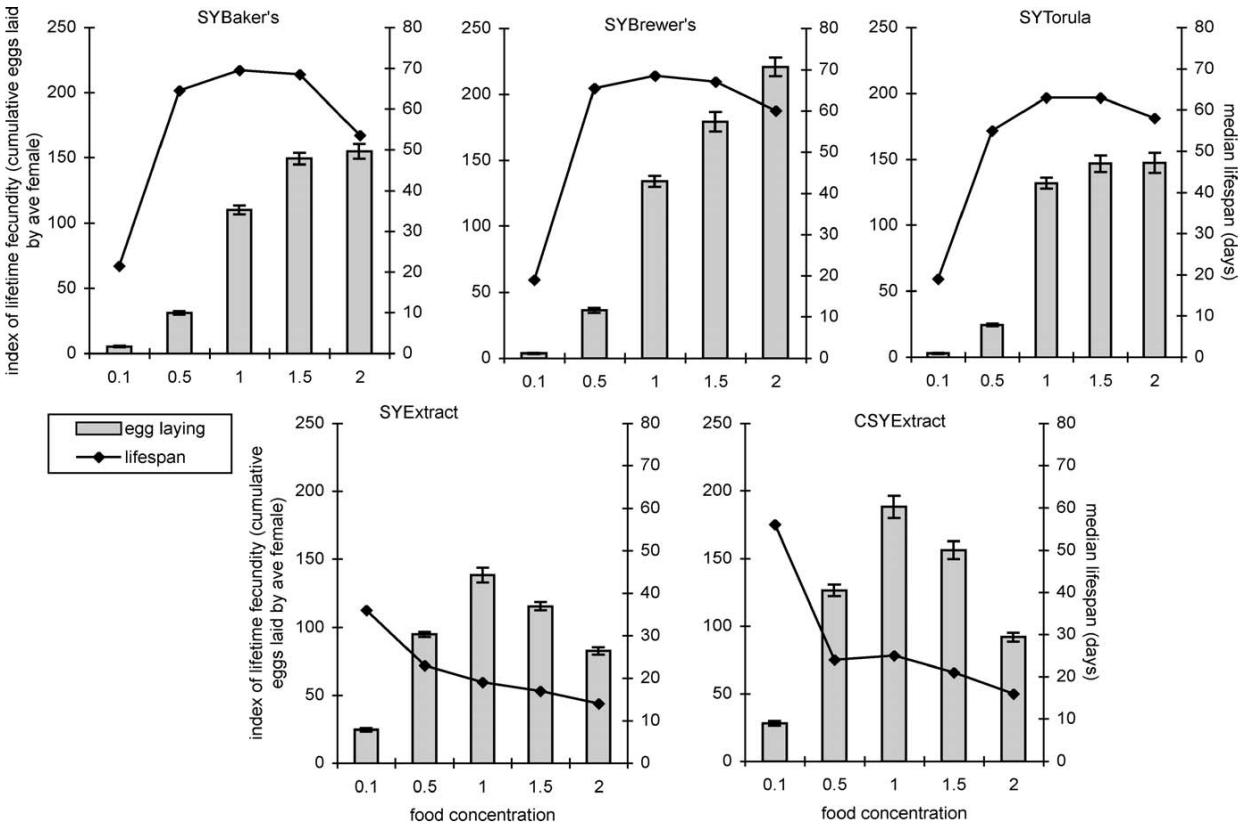

Figure 2. Effect of a range of concentrations of different commercially available yeasts on life span and fecundity. Five different yeast concentrations were prepared for each of five different sugar/yeast (SY) recipes. SYBaker's, SYBrewer's, and SYTorula each refer to food made with different, inactivated whole-yeast preparations, whereas SYExtract and CSYExtract refer to diets based on a water-soluble yeast extract. The nutritional components in each food type were sucrose and yeast or yeast extract and cornmeal (for CSYExtract only). *Bars:* index of lifetime fecundity ± standard error of the mean; *connected black points:* median life-span values. We specified a linear model to describe fecundity (Materials and Methods), which found all factors and interactions to be significant. The predicted values are plotted against observed values in Supplementary Figure 2. Each food concentration range was performed once, except for SYBaker's and SYBrewer's, which were performed twice.

Varying the Quality of the Yeast Supply Produces a Range of Effects on Life Span and Fecundity

The above data and (32) show that DR in *Drosophila* is achieved solely by modulating the yeast component of the diet. We next compared a variety of different yeasts to determine their effects on life span and fecundity. These experiments included four sources of inactivated yeast: a baker's yeast, a brewer's yeast, a torula yeast, and a water-soluble extract of baker's yeast. The first three of these yeasts are whole-cell lysates, whereas the fourth is a purified extract. Each of the yeasts was used over a range of concentrations from 10 g/L (labeled 0.1) to 200 g/L (labeled 2.0) while the other media constituents were held constant (Table 1).

Comparison of the three whole-yeast food types (SYBaker's, SYBrewer's, and SYTorula) showed a similar pattern for median life span, with a peak at 1.0 (100 g/L) and a decline as food concentration was changed above or below this point (top three graphs of Figure 2). SYBaker's and SYBrewer's yielded the longest life spans (69- and 70-day medians, respectively, on 1.0 food), whereas the longest life span on SYTorula (63-day median at 1.0) was significantly shorter ($p < .0001$ in both comparisons, log-rank test). For each of these three yeasts, lifetime fecundity increased with increasing food concentration to 1.5, above which there was no further increase for SYBaker's and SYTorula, but there was for SYBrewer's when the concentration was raised from 1.5 to 2.0 (Supplementary Figure 2). Furthermore, the level of egg laying on 2.0 SYBrewer's was higher than the peak value for any of the other food types tested. Thus, the observed limit to egg-laying on the other food types was not intrinsic to the physiology of the flies, but was restricted by some feature of the foods. In other experiments we have also raised the yeast concentration in SYBrewer's medium to 300 g/L (3.0) and saw a further life-span shortening from 2.0 ($p < .05$, log-rank test). However, this was not accompanied by a further increase in fecundity beyond the level in 2.0 ($p = .53$, Wilcoxon rank sum test; Supplementary Figure 3).

The flies responded differently to the yeast-extract–based media. The most obvious difference was that life span decreased for each addition of yeast extract to the medium. This was similar for CSYExtract and SYExtract (bottom

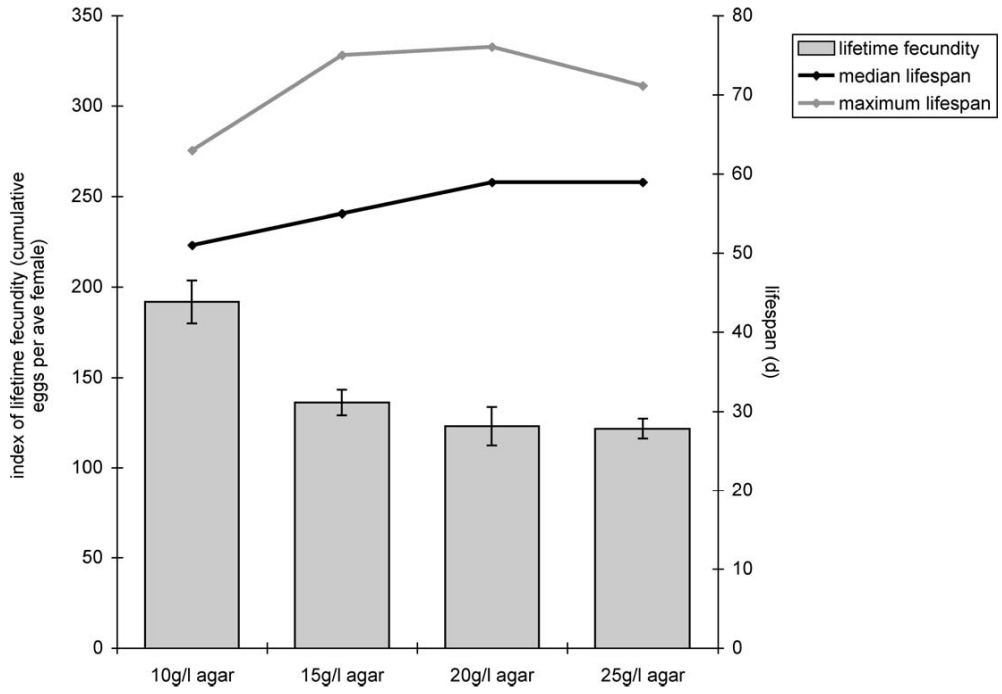

Figure 3. Effect of varying agar concentration on life span and fecundity of females on SYBrewer's medium. The effect of food hardness on life span and fecundity was tested by altering the agar concentration while all other ingredients were held at fixed concentrations (Table 1). This medium contained Brewer's yeast at 200 g/L (2.0 level) (agar concentration ranges were also tested at two other SYBrewer's concentrations; data not shown). *Bars:* index of lifetime fecundity ± standard error of the mean; *connected black points:* median life span; *connected gray points:* maximum life span (median of the last 10% survivorship).

two graphs of Figure 2), except for 0.1, at which level cornmeal addition resulted in a significantly longer life span (36 days on SYExtract vs 56 days on CSYExtract; $p < .0001$, log-rank test). Because the positive effect of cornflour on life span was only seen at the lowest concentration of yeast extract (0.1) and the longest life span on 0.1 SYExtract was low compared with all other treatments, the data are compatible with an argument that yeast extract caused dose-dependent toxicity. The pattern of lifetime fecundity was similar between SYExtract and CSYExtract, increasing with yeast extract addition to a maximum at 1.0, but decreasing at higher concentrations. Cornmeal addition augmented egg laying, which peaked in 1.0 CSYExtract at a level similar to that in 1.5 SYBrewer's and higher than the maxima for the other three food types. In both the presence and absence of cornmeal, yeast extract was apparently more nutritionally dense than whole-yeast powders, because egg laying was greater on CSYExtract (up to 1.0) and SYExtract (up to 0.5) than on the whole-yeast diets at corresponding food concentrations. However, fecundity decreased for additions of yeast extract higher than 100 g/L (1.0). Thus, yeast extract at high concentrations is detrimental to fecundity in addition to negatively affecting life span.

Is DR in Drosophila *a Nutritional Response?*

In order to fulfill the requirements for DR, it is necessary that the longer-lived (restricted) animals are not simply less sick than those with higher nutritional intake. One indication of this comes from increased fecundity with increasing nutrients. However, if the food delivers both nutrients to benefit fecundity as well as a toxic effect that reduces life span, the phenotype would be indistinguishable from a true effect of DR (30). It is therefore important to try and distinguish directly between a toxin-based and a nutrient-based explanation for the life span–shortening effect of the high nutrient concentration.

Increasing the food concentration could mimic a DR effect by increasing the hardness of the food. To test this possibility, we fixed the concentration of all food ingredients (at 2.0 SYBrewer's) and varied the agar concentration on its own (Figure 3). For each increase in agar concentration, there was a trend toward a decrease in lifetime fecundity. However, this trend was only significant for the increase from 10 g/L to 15 g/L ($p < .0005$, Wilcoxon rank sum test). This reduction was accompanied by a significant increase in life span when the agar concentration was raised from 10 g/L to 15 g/L ($p < .01$, log-rank test) and a further, nonsignificant ($p = .09$, log-rank test) increase when agar was raised to 20 g/L. These data are consistent with agar controlling food availability in a nondetrimental way between 10 g/L and 20 g/L agar, and is therefore explicable as a DR effect. When the agar concentration was further increased to 25 g/L there was no change in median life span or lifetime fecundity, but maximum life span decreased from a median of 76 days to 71 days (Figure 3). This result argues that older flies do indeed differentially suffer if the food

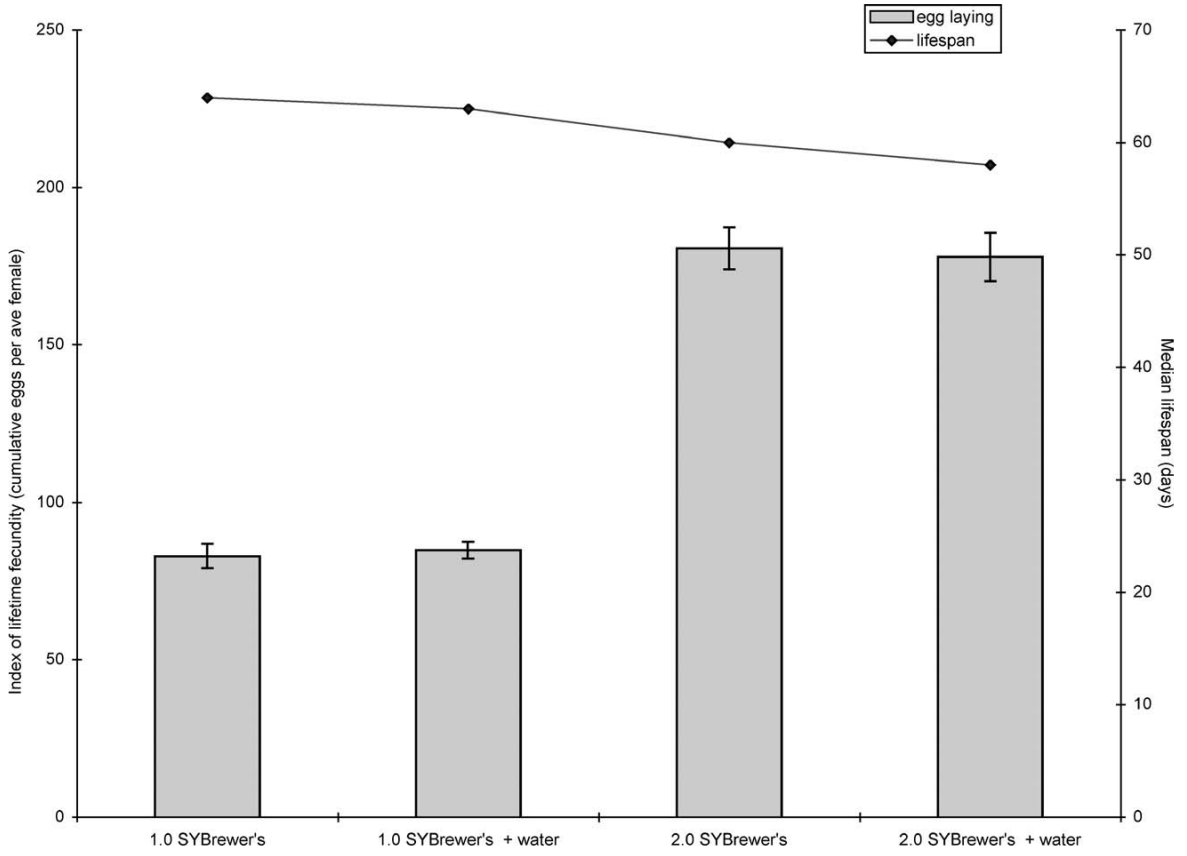

Figure 4. Effect of water addition on the dietary restriction (DR) response of flies on SYBrewer's medium. Free access to water was provided in the form of 1% agar in a pipette tip inserted into the food. *Bars:* index of lifetime fecundity ± standard error of the mean; *connected points:* median life span. Experiment was performed twice.

becomes sufficiently hard, but for agar concentrations < 20 g/L food hardness does not on its own cause the life-shortening (DR) effect seen in Figure 2. We also tested the effect of agar concentration for 1.0 SYBrewer's and 3.0 SYBrewer's (data not shown). Although qualitatively similar, this experiment also revealed an interaction with the yeast concentration, whereby flies were more sensitive to higher agar concentrations at higher yeast concentrations. This result indicates that the yeast content of the food can contribute to overall food hardness and adversely affect life span.

Another possible detrimental effect of high food concentrations concerns water availability, because the food is the only source of water. We therefore tested if water addition could overcome the adverse effects of high nutrition levels on life span. Figure 4 shows that addition of a fresh source of water to 1.0 and 2.0 SYBrewer's could not rescue the life-shortening effect of high nutrient concentrations and had no effect on lifetime fecundity. Therefore, inability to access sufficient free water does not explain the life span–shortening effect accompanying high nutrient concentrations in the food.

Discussion

DR is a well-established intervention for extending fly life span. Indeed, the interaction among diet, life span, and fecundity has formed the basis for both practical and theoretical investigations into the possible trade-offs between these life-history traits (39). Here we have investigated DR more closely and found that, without careful attention to the food composition, studies that claim to be examining extended life span due to DR may simply be studying the rescue of normal life span from the effects of inappropriate food types that prematurely shorten life. It thus follows that any mechanistic conclusions drawn from such studies are likely to be obscured by the detrimental effects of the food and so would be inappropriate to address questions of how DR operates to preserve life span for *Drosophila* or other species.

Drosophila in the wild is thought to coconsume fruit material and microbes from fermenting and/or rotting fruit (40). In the laboratory, *Drosophila* can be maintained on a combination of sugar, yeast, and water (35). We found that addition of sugar > 50 g/L to the culture medium was detrimental for egg laying and that variations from 0 to 150

g/L had little effect on life span (Figure 1). These data indicate that *Drosophila* has a very low requirement for free sugar for maximal life span and fecundity, consistent with the finding that total sugar levels in rotting banana are no more than 20 mM (equivalent to 4.5 g/L sucrose) (41). Other experiments have shown that *Drosophila* modulate their feeding behavior only slightly, or not at all, when sucrose levels rise above 50 g/L (32,42,43). Thus, the dramatically lowered egg laying observed with high sugar is unlikely to be an effect of reduced feeding in response to the altered sucrose concentration, and instead probably reflects an adverse effect on physiology due to the presence of unnaturally high sugar levels. These data show that high sugar should be avoided in *Drosophila* DR experiments.

In contrast, increasing additions of one particular brewer's yeast caused lifetime fecundity to continually increase over a concentration range that also decreased life span and so conformed to the expectations of a DR treatment. When recently changing our yeast supplier, we noted a shift in the concentration at which life span peaked from 65 g/L yeast [0.65 in (32)] to 100 g/L (1.0 shown here). Yeast quality is thus highly variable. Furthermore, high yeast concentrations that reduce life span are not always associated with increasing fecundity. This fact is at odds with the recognized effect of DR on fecundity in worms (22) and rodents (31), and is consistent with an explanation that the life-span decrease on high food concentrations is not an effect of increased nutrition, but due to some detrimental effect of the yeast composition. This could be caused by either a direct effect of a specific toxic element whose increasing concentration reduces life span and perhaps also fecundity or an indirect effect of a nutritionally imbalanced diet that results in ill health.

Under the first explanation, one would expect a pattern of fecundity and life span similar to that seen for the flies fed increasing concentrations of yeast extract. In this situation, both nutrients and the toxin (e.g., a heavy metal) are delivered in the food. This situation results in increasing fecundity as nutrients increase and toxicity remains below a tolerable threshold (e.g., 1.0 in SYExtract and CSYExtract in Figure 2), beyond which fecundity is reduced. For this same concentration range, life span would be ever decreasing. This explanation is consistent with data for *C. elegans* grown on different types of bacteria. It is currently common practice to grow worms on *Escherichia coli*, which can support growth and reproduction and upon dilution elicit an apparent DR response (22). However, when the worms are grown on the soil bacterium *Bacillus Subtilis*, their life span is increased some 50% without changes in development time or reproductive output (26). Thus, any nutrient-dependent life-span shortening when increasing the concentrations of *E. coli* for worms or yeast extract for flies would be combined with the effects of food toxicity.

In contrast, nutritional imbalance would be expected to yield a life-history pattern like that for SYBaker's and SYTorula, where the absence of a nutritional component imposes a limit on egg-laying capacity due to depletion from parental reserves. Previous data on the nutritional requirements of adult *Drosophila* showed that deficiencies for essential amino acids, chloride, phosphorous, or calcium reduced egg laying within 16 days, with little effect on the short-term viability of the adult (44). Thus a trace element shortfall may limit lifetime egg-laying capacity with little effect on immediate risk of death. An example of this phenotype is shown for flies on 1.5 and 2.0 SYBaker's, which have the same level of lifetime fecundity but markedly different life spans (Figure 2). Because they both experience the same limitation to lifetime fecundity, the limitation in itself is not what causes shortened life span on 2.0. Rather, the increasing excess of other dietary components, and so nutrient imbalance, is the most likely explanation for the elevated mortality.

In an attempt to identify any such toxins or nutrient imbalances, we have compared the available nutritional data for each of the yeast types used (Supplementary Table 2). Unfortunately, these analyses have a limited scope because only standard nutritional constituents are measured; therefore, many potentially toxic compounds will be overlooked. It is possible, however, to compare nutrient ratios among yeasts. In this light it is notable that several vitamins are an order of magnitude lower in concentration in SYBaker's than in SYBrewer's. These vitamins include biotin, a deficiency of which is thought to shorten *Drosophila* life span (45). This could be tested by the addition of these vitamins to the food to see if they rescue fecundity and affect life span. As a note, it is possible that similarly subtle effects of food type belie unknown nutrient imbalances in DR experiments that have been performed in other model organisms. For example, rescue from a nutrient imbalance could explain the life-span extension found in rats when the dietary protein source casein was replaced with soy protein (12). Subtle differences in food affecting life span have also been demonstrated by experiments on mice and rats subjected to methionine restriction (15,17,46). Thus, diet optimization is also an important consideration for DR studies in rodents, in which food composition varies depending on the particular commercially available chow that is used.

Despite all these precautions to establish a diet suitable for *Drosophila* DR, it is still possible that the food could have a detrimental effect on life span unrelated to nutrition and with no adverse effect on lifetime fecundity, thus mimicking the DR effect. Because we use a food dilution method for DR, the hardness of the food and water availability are the most likely candidates to produce such an effect. Our experiments showed that neither could account for the life-span shortening seen when varying the yeast concentration. We did note, however, a detrimental effect on maximum life span when agar concentration was raised to an extremely high level (25 g/L, more than twice that used for our other experiments). This effect was exacerbated when the yeast concentration was also raised to 300 g/L, showing that food hardness can reduce *Drosophila* life span. This non-DR–based life-shortening effect of hard food is likely to have contributed heavily to the life-span shortening seen in studies when yeast and sugar are both raised to 300 g/L and agar to 20 g/L (34,47) (Supplementary Figure 3).

Based on the data presented above, we conclude that the brewer's yeast is the most suitable of those that we tested

for DR studies and it now forms the basis for our laboratory recipes. This change has the additional advantage of bringing the nutritional content of our fly diet in line with that of two other laboratories studying fly DR using the same yeast (Helfand and Pletcher laboratories, Scott Pletcher, personal communication, 2005). We are now in the process of extending this study by applying this DR regimen to male flies as well as a variety of commonly used laboratory strains of *Drosophila*. As there is an impact of genotype on the fly response to DR (48), it will be interesting to see if other laboratory strains (both inbred and outbred) exhibit a similar response to these foods. Modulations or even loss of the DR response in these lines may be informative about the mechanisms of DR.

Conclusion

This work highlights the need for validated diets used for DR as a step toward establishing some dietary uniformity in the DR community to allow direct comparison of different experiments with the same species and of different species. For flies, the dramatic variability in quality of yeasts from different suppliers, and presumably between seasons, points to the need for a defined synthetic medium that would avoid the potential problems of unwanted detrimental effects being introduced into *Drosophila* experiments from the yeast or its feedstock.

Acknowledgments

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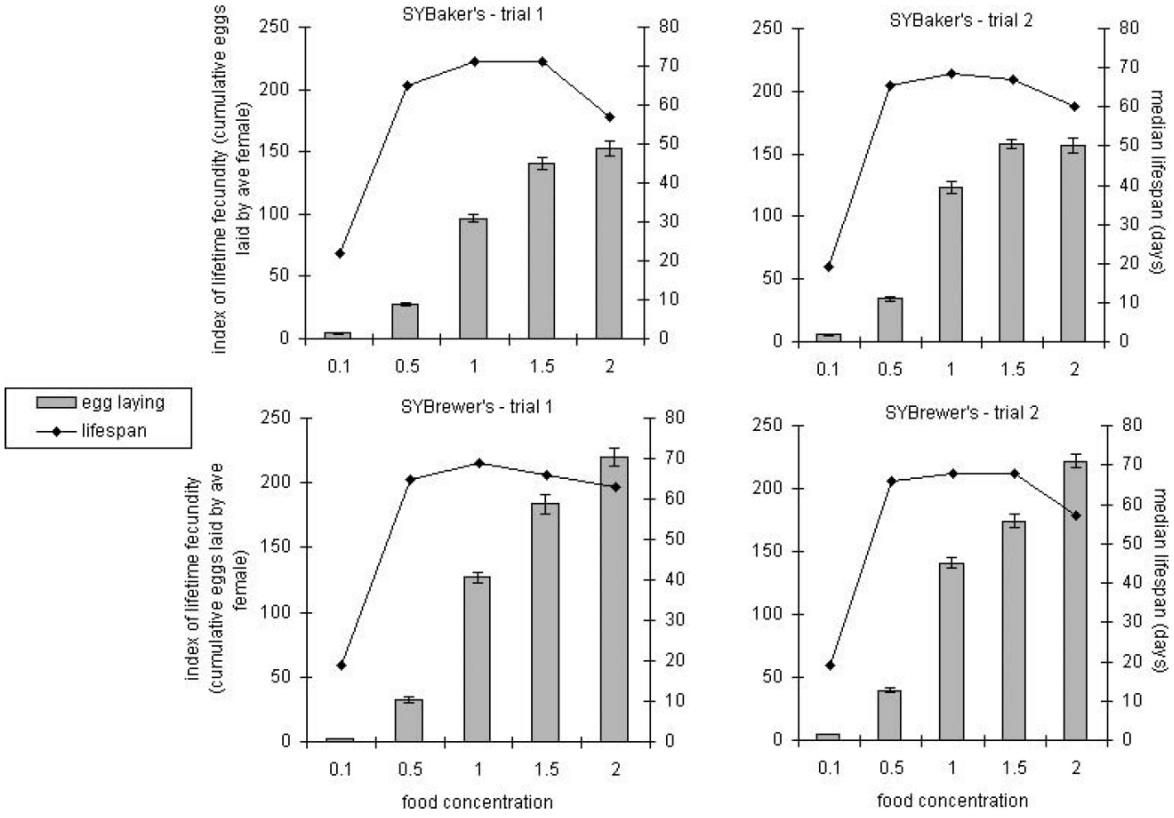
Supplementary Figures and Tables



Supplementary Figure 1. Data for both trials run for SYBaker's and SYBrewer's. These are independently replicated data sets from nonoverlapping generations of flies.

Supplementary Table 1. Life Span and Fecundity Data for Survivorships Referred to in Figures

Condition	Lifetime Fecundity*	(*SEM*)	Median LS	Mean LS	(*SEM*)
Figure 1					
0 g/L sucrose	79.4	(4.3)	47	42.5	(1.4)
50 g/L sucrose	80.2	(4.6)	54	52	(1)
100 g/L sucrose	45.3	(3.3)	54	55	(1.1)
150 g/L sucrose	46.9	(3.5)	54	52	(1.3)
Figure 2 and Supplementary Figure 1					
0.1 SYBaker's – Trial 1	5.9	(0.6)	21	21.9	(0.5)
0.5 SYBaker's – Trial 1	34.2	(1.4)	64	62.7	(1.1)
1.0 SYBaker's – Trial 1	123.3	(4.6)	68	67.0	(0.8)
1.5 SYBaker's – Trial 1	158.2	(3.7)	66	64.7	(0.9)
2.0 SYBaker's – Trial 1	157.0	(5.9)	50	49.4	(1.6)
0.1 SYBaker's – Trial 2	4.4	(0.5)	22	24.8	(1.2)
0.5 SYBaker's – Trial 2	27.9	(1.5)	65	62.8	(1.3)
1.0 SYBaker's – Trial 2	96.7	(3.3)	71	68.8	(1.2)
1.5 SYBaker's – Ttrial 2	140.4	(4.5)	71	68.3	(1.1)
2.0 SYBaker's – Trial 2	152.6	(5.7)	57	54.5	(1.4)
0.1 SYBrewer's – Trial 1	2.4	(0.3)	19	21.0	(0.8)
0.5 SYBrewer's – Trial 1	32.5	(2.0)	65	63.3	(1.2)
1.0 SYBrewer's – Trial 1	126.8	(4.2)	69	67.8	(0.9)
1.5 SYBrewer's – Trial 1	183.9	(7.4)	66	65.2	(1.2)
2.0 SYBrewer's – Trial 1	219.5	(6.9)	63	62.2	(1.1)
0.1 SYBrewer's – Trial 2	4.7	(0.2)	19	19.8	(0.4)
0.5 SYBrewer's – Trial 2	39.8	(1.6)	66	62.8	(1.2)
1.0 SYBrewer's – Trial 2	140.9	(4.0)	68	66.9	(0.5)
1.5 SYBrewer's – Trial 2	174.2	(5.2)	68	67.1	(0.6)
2.0 SYBrewer's – Trial 2	221.9	(5.4)	57	59.0	(0.8)
0.1 SYTorula	2.8	(0.3)	19	21.3	(0.8)
0.5 SYTorula	24.4	(1.1)	55	53.2	(1.2)
1.0 SYTorula	131.9	(4.2)	63	61.4	(1.0)
1.5 SYTorula	146.5	(6.1)	63	63.4	(1.1)
2.0 SYTorula	147.2	(7.6)	58	55.9	(1.4)
0.1 SYExtract	24.5	(1.3)	36	36.4	(1.4)
0.5 SYExtract	94.6	(1.9)	23	23.4	(0.6)
1.0 SYExtract	138.2	(5.4)	19	19.7	(0.3)
1.5 SYExtract	115.4	(3.0)	17	17.1	(0.2)
2.0 SYExtract	82.5	(2.9)	14	14.1	(0.2)
0.1 CSYExtract	27.8	(1.8)	56	54.4	(1.4)
0.5 CSYExtract	126.3	(4.2)	24	25.4	(0.7)
1.0 CSYExtract	188.1	(8.3)	25	25.2	(0.8)
1.5 CSYExtract	156.2	(6.5)	21	21.1	(0.4)
2.0 CSYExtract	91.8	(3.3)	16	16.0	(0.2)
Figure 3					
10 g/L agar	191.6	(11.8)	51	50.8	(1.5)
15 g/L agar	136.3	(7.0)	55	55.3	(1.8)
20 g/L agar	123.2	(10.6)	59	58.8	(1.6)
25 g/L agar	121.8	(5.5)	59	59.8	(1.1)
Figure 4					
1.0 SYBrewer's – water	82.9	(3.9)	64	63	(1.1)
1.0 SYBrewer's + water	84.8	(2.7)	63	61.2	(1.5)
2.0 SYBrewer's – water	180.6	(6.6)	60	59	(1.3)
2.0 SYBrewer's + water	177.8	(7.7)	58	53.9	(1.0)
Supplementary Figure 3					
1.0 SYBrewer's	57.9	(8.0)	67	66.4	(2.7)
2.0 SYBrewer's	136.3	(7.0)	55	55.3	(1.8)
3.0 SYBrewer's	141.6	(6.5)	53	51.5	(1.4)

Notes: *Lifetime fecundity is the sum of eggs laid by an average female on the days of counting.
SEM = standard error of the mean; LS = life span.

Supplementary Table 2. Nutritional Comparison of the Different Yeasts Tested

	Nutrient Composition (g/100 g dry weight)			
Nutrient	Baker's Yeast*	Brewer's Yeast*	Torula Yeast	Bacto Yeast Extract*
Carbohydrates	39	35	28	†
Protein	45.7	45	58	51
Fat	5.8	1	7	
Alanine‡	3.97		7.3	5.6
Arginine	2.01	5.1	5.6	2.6
Asparagine/Aspartate	4.2		10.6	5.3
Cysteine (variable)	0.12	1.2	0.4	Destroyed§
Glutamine/Glutamate	9.1		13.6	9.4
Glycine	1.41	4	4.9	3
Histidine	0.89	2.5	2.2	1.3
Isoleucine	1.68	4.1	5.6	3
Leucine	2.52	5.7	8.4	4.1
Lysine	2.48	6.2	8.8	4.6
Methionine	0.5	1.2	1.7	0.8
Phenylalanine	1.48	3.1	5.1	2.6
Proline	1.41		4.2	2
Serine	1.9		5.7	
Threonine	2.07	4.4	5.8	1.6
Tryptophan	Destroyed	1.1	0.9	Destroyed
Tyrosine	0.99	3.2	4	1.2
Valine	1.89	4.8	6.1	3.5
Inositol		0.47		
Choline		0.47		
Cadmium			0.00004	
Calcium	0.134	0.12	0.4	0.013
Chloride	1			0.38
Cobalt		0.00015		
Copper	0.0002	0.0035	0.0008	
Iron	0.0133	0.02	0.0125	
Lead			0.00002	
Magnesium	0.262		0.1	0.075
Manganese		0.00053		
Phosphate				3.27
Phosphorous	1.603	1.5	0.9	
Potassium	2.447	0.86	1.6	3.195
Sodium	0.041		0.02	1.49
Sulfate				0.09
Sulfite			0.018	
Zinc	0.0062	0.00387	0.01	
Biotin	0.0000236	0.000125		
Ca Pantothenate	0.00211	0.0122		
Folic Acid	0.000871	0.0005		
Nicotinic Acid	0.0182	0.04		
Pyridoxine	0.00046	0.005	0.0425	
Riboflavin	0.00103	0.0045	0.006	
Thiamine-Hcl (aneurin)	0.00067	0.015	0.0002	

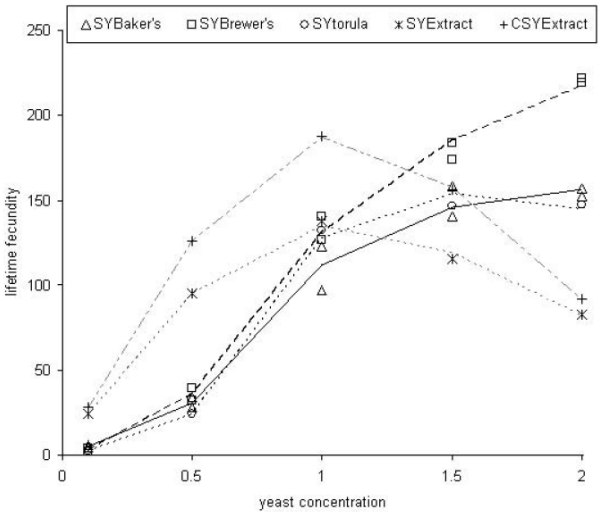
Notes: Each of the analyses above are provided by the manufacturers and describes a typical batch, except for that of baker's yeast, the nutritional breakdown of which was not supplied by the manufacturer. This information was gathered independently using a sample from a bag used in our laboratory for these experiments.

*These yeasts are labeled *Saccharomyces cerevisiae*.

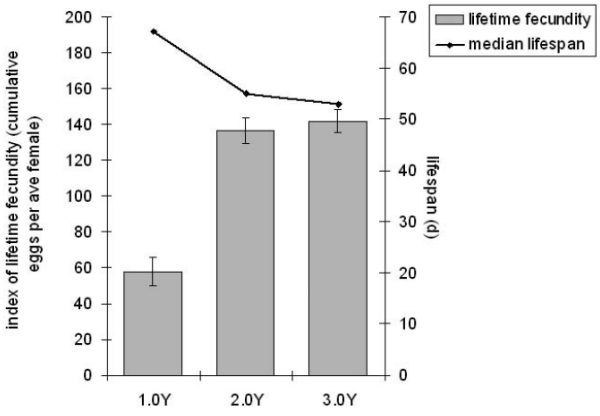
†Missing values are indicative of data not reported, not that the nutrients are absent.

‡Amino acids are reported as total amino acid content. Values for free soluble amino acids are lower.

§Indicates components lost or destroyed by the detection/quantification process.



Supplementary Figure 2. Model predictions versus actual egg-laying data reported in Figure 2. Model predictions are represented by the *lines* and actual data by *symbols*. All fixed terms (yeast type, concentration and the quadratic term for concentration) and interactions were significant.



Supplementary Figure 3. Effect of raising yeast concentration in SYBrewer's above the range used for dietary restriction (DR). Yeast concentration was raised to 300 g/L, and life span and egg laying were monitored. Whereas life span showed a significant decline from that found at 2.0 SYBrewer's, egg laying was not further increased, indicating that the flies did not experience a higher level of nutrition. Agar concentration was 15 g/L.

Appendix 2: Grandison *et al*. (2009)

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Effect of a Standardised Dietary Restriction Protocol on Multiple Laboratory Strains of *Drosophila melanogaster*

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Abstract

Background: Outcomes of lifespan studies in model organisms are particularly susceptible to variations in technical procedures. This is especially true of dietary restriction, which is implemented in many different ways among laboratories.

Principal Findings: In this study, we have examined the effect of laboratory stock maintenance, genotype differences and microbial infection on the ability of dietary restriction (DR) to extend life in the fruit fly *Drosophila melanogaster*. None of these factors block the DR effect.

Conclusions: These data lend support to the idea that nutrient restriction genuinely extends lifespan in flies, and that any mechanistic discoveries made with this model are of potential relevance to the determinants of lifespan in other organisms.

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Introduction

In order to maximise its genetic contribution to posterity, an organism must appropriately direct the use of nutrients to traits such as growth, reproduction and repair. In some circumstances, this will mean maximising one trait at the expense of another. This idea has been used to explain the observation that relatively low food intake can result in longer life, because it comes at the cost of reduced rates of reproduction [1–4]. This particular trade-off phenomenon is widespread and has been termed dietary restriction or DR. Although extensively studied since its first description in 1935 [5], very little is known about the molecular details of exactly what resources are shared in this trade-off and how they are balanced between the traits. Uncovering these mechanisms has now become the holy grail of research into DR, with the aim of harnessing their power for longer and healthier lives.

One of the promising advances towards the goal of uncovering the mechanisms by which DR extends life was the discovery that the effect is evolutionarily conserved [6–10]. However, even with the use of short-lived model organisms for relatively rapid lifespan experiments, the mechanisms remain elusive. This is likely to be largely due to the complexity of physiology involved in determining length of life, but may be also in part due to technical issues in experimental design hampering a clear path of progress [11]. The ease with which complexity can be introduced into these studies can be illustrated by the large effects on fly lifespan caused by very small changes in nutrition. For example, substituting one source of the dietary yeast *Saccharomyces cerevisiae*, with another from a different supplier in an otherwise identical diet can have large effects on fly lifespan [12]. Similarly, lifespan differences have been reported due to the use of different bacterial strains as food for *Caenorhabditis elegans* [13] or by interchanging casein and soy peptone as the source of dietary protein for rodents [14]. In fact, a recent article has proposed that DR itself may have arisen as a by-product of laboratory life as animals are unintentionally subjected to selective breeding in the presence of an artificially rich nutritional environment [15]. Clearly, these issues need to be addressed if we are to uncover the molecular mechanisms of DR.

In our studies on DR in *Drosophila*, we have taken a systematic approach to optimise dietary composition such that fecundity and lifespan are maximised and any non-specific adverse effects of the food are avoided [12]. In this article, we extend this work to examine the effect of different techniques of long-term stock maintenance and microbial infection on the responses of 'wild-type' laboratory-maintained flies to DR. We have undertaken these experiments in order to establish a working protocol that avoids laboratory artefacts and will therefore aid studies seeking the molecular mechanisms of DR. As a result of performing these experiments with flies of different genetic backgrounds, we find interesting differences in the interaction between diet and genotype that form a solid basis for future work to uncover how DR extends the lifespan of flies and other organisms.

Results

An intermittent feeding regime did not affect Drosophila lifespan

We have previously published a description of the optimisation of a sugar/yeast (SY) medium for DR studies in flies [12]. This

study found that yeast dilution in an otherwise unchanged medium effectively limits the flies' nutrient intake, decreases their daily and lifetime fecundity and increases their lifespan.

An alternative DR protocol that extends rodent lifespan is every other day feeding (EOD) [16,17]. In these experiments, the EOD cohort has alternating bouts of 24 h access to unlimited food followed by 24 h starvation, while controls have continuous access to unlimited food. Interestingly, this intervention extends lifespan even though the EOD animals nearly fully compensated for the periods of starvation by eating more. Thus, intermittent periods of starvation could be equally as important as reduced nutrient intake for extending lifespan.

Two *Drosophila* studies have attempted a similar regimen and one reported a generally positive effect on lifespan when flies were subjected to 18 h access to food and 6 h access to water only in every 24 h [18]. In contrast, a more recent study has reported no positive effects of this treatment, or of any other treatments in which the timing of the starvation/feeding periods was altered [19]. However, in this latter study, the treatment was only implemented on 5 out of every 7 days of adult life, making it possible that any beneficial effects of the protocol were masked by the days without treatment. We therefore decided to test this technique using our laboratory strain Dahomey, applying daily bouts of either 3 h or 6 h starvation, during which the flies had access to water only. We found that neither treatment had a positive or negative effect on lifespan (Figure 1). While this could be taken to mean that DR does not work in flies, the lack of any effect on lifespan of the more severe restriction makes it impossible to know to what extent the flies were nutrient restricted or whether the periods of starvation were close to adequate to elicit a protective effect. Without a more extensive set of starvation periods, it is not possible to draw definitive conclusions about the effectiveness of this intervention in *Drosophila*.

Comparison of the DR response between different laboratory strains

In all of our DR optimisation experiments we have used our outbred laboratory strain of *Drosophila*, Dahomey. This strain has been maintained for many years on an SY diet in large population cages with overlapping generations. In contrast, most laboratory wild-type strains are largely inbred and maintained in relatively small numbers in individual containers and may have a varied nutritional history. Some of these housing conditions can easily lead to selection for early reproduction, which is known to cause shortened lifespan [20–22]. We therefore assayed the lifespan of several commonly used wild-type *Drosophila* strains on our standard SY food (1×; Figure 2). In all cases, the lifespans were significantly shorter than that of Dahomey and exhibited median lifespans from 53 days for OregonR to 65 days for Dahomey.

Next we asked what the effect of this variation was on the DR response in these different strains. This was both to assess how our DR protocol is likely to behave when implemented in other laboratories that routinely use fly stocks other than Dahomey, as well as to look for strains with altered DR responses that might provide insights into its mode of action. The operational definition of DR is the range of nutrition that causes lifespan to increase and fecundity to decrease [23]. It should be noted that this definition excludes the dilution from 0.5× down to 0.1×, as this caused the flies to become malnourished and both lifespan and fecundity to decrease (Figure 3). For Dahomey and wDahomey, the DR range was from 2× to 0.5× food, while for w1118 and CantonS it was from 2× to 1×, and for OregonR was from 1.5× to 0.5× (Figure 3). For OregonR only, the highest food concentration caused egg laying to decrease, which indicated that the associated lifespan decrease from 1.5× to 2× was not accompanied by increased intake of biologically valuable nutrition and therefore could be due to a non-specific detrimental effect of high food. It was thus considered outside of the functional DR range for this strain. Finally, for yw, there was a clear DR response from 1× to 0.5× food but, owing to incomplete data, we cannot report any possible broader DR effect. Thus in all cases, a DR response was observed under these conditions although its exact nature was different for different wild-type strains.

In all comparisons from all trials, Dahomey, wDahomey and yw exhibited the longest lifespan (Table 1) with medians from 69 to 73 days over different trials on 0.5× food (Dahomey v wDahomey, $p = 0.69$; Dahomey or wDahomey v highest median lifespan from each other genotype, $p<0.001$, log-rank test). Dahomey and wDahomey also exhibited higher reproductive output than the other wild-types at each food concentrations except 0.1×, as well

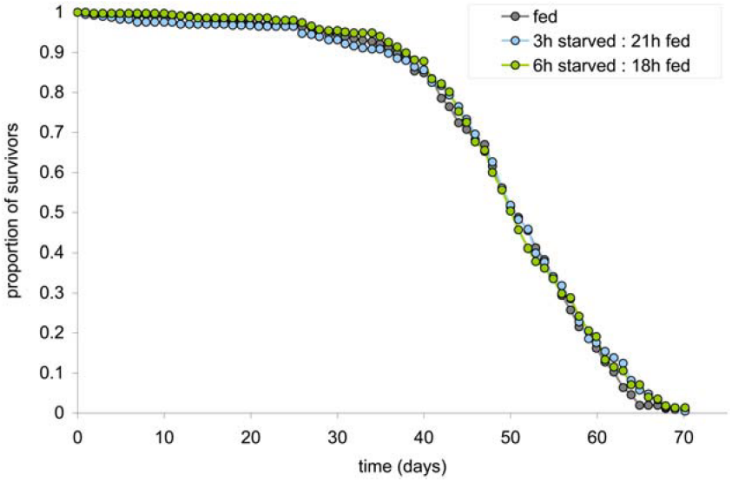

Figure 1. Intermittent exposure of flies to food does not increase their lifespan. Throughout adult life, Dahomey females were exposed to daily cycles of starvation:feeding of either 3 h:21 h or 6 h:18 h. Neither treatment had any effect on lifespan. During the periods of starvation, flies had access to water only.
doi:10.1371/journal.pone.0004067.g001

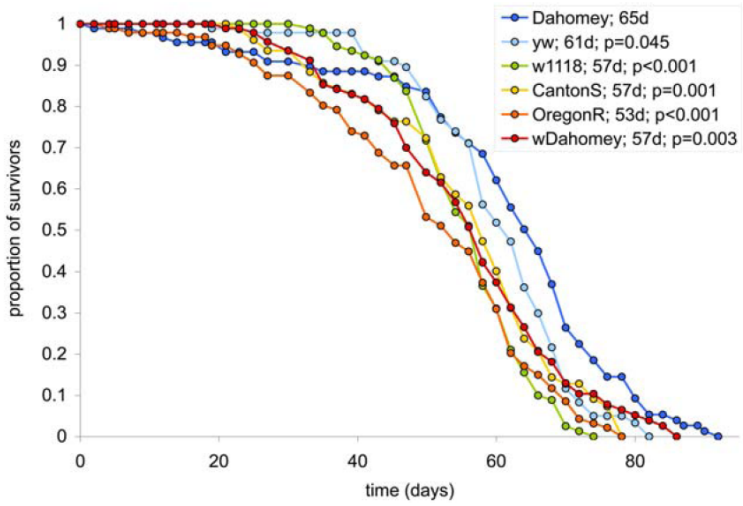

Figure 2. Different laboratory strains of wild-type Drosophila have different lifespans. Each genotype was raised in parallel under the same conditions and assayed on 1× SY for lifespan. All strains that were tested exhibited a shorter lifespan than our outbred laboratory strain Dahomey. The graph legend reports the strain name; median lifespan in days and; p-value from the log-rank test when compared to Dahomey.
doi:10.1371/journal.pone.0004067.g002

as the maximum reproductive output from all conditions (on 2× food) (Dahomey v wDahomey, $p = 0.97$; Dahomey or wDahomey v highest reproductive output for each other genotype, $p < 0.003$, Wilcoxon rank-sum test).

Effect of tetracycline treatment on the DR effect

Drosophila are host to a range of microbes, and for many strains, this includes a bacterium of the genus *Wolbachia* that resides in the cytoplasm of reproductive tissues [24]. In some cases, the presence of *Wolbachia* has been shown to alter lifespan [25]. Recently, a vertically inherited factor that was curable by tetracycline treatment was shown to account for at least part of the long lifespan of a long-lived *Drosophila* mutant [26]. We decided it was important to examine the effect of such infections on DR, because if they account for the lifespan difference, it is unlikely DR in *Drosophila* is useful as a model for higher organisms.

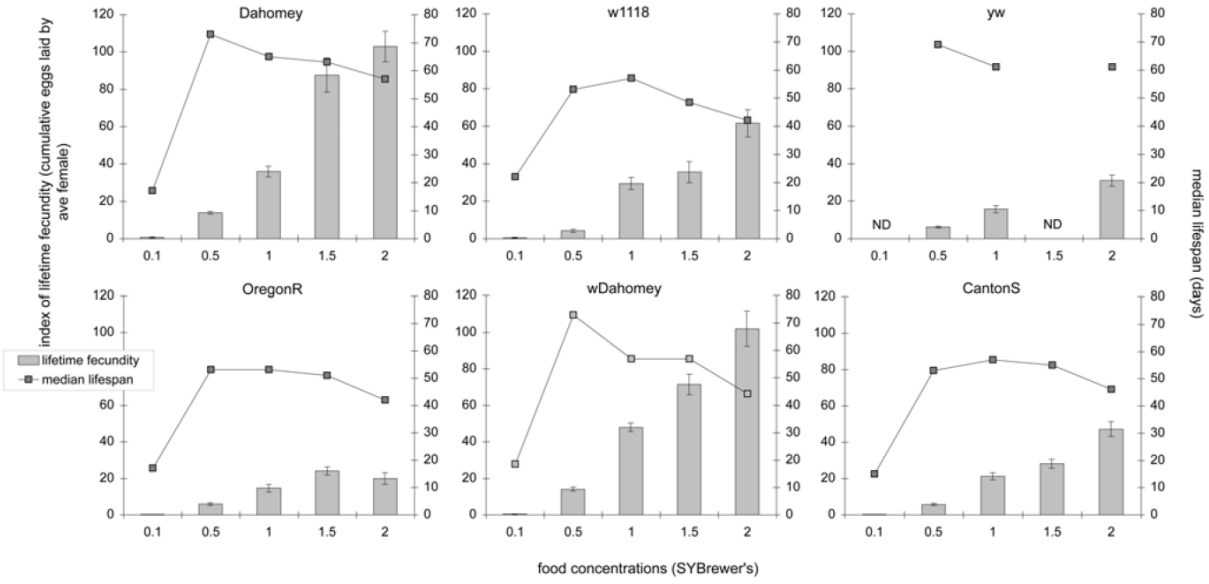

Figure 3. Different laboratory strains subject to DR. When tested in parallel under the same conditions, all wild-type strains tested exhibited a DR response. This is defined as a simultaneous increase in lifespan and decrease in lifetime fecundity when nutrient availability was reduced. Bars: index of lifetime fecundity±standard error of the mean; connected points: median lifespan in days; ND: not determined. Data shown are from a single trial in which all lifespans were run simultaneously. They are representative of triplicate data sets for Dahomey, CantonS and OregonR and duplicates for yw; w118 and wDahomey data are from a single trial.
doi:10.1371/journal.pone.0004067.g003

Table 1.

Wild-type strain	Food Conc (x)	Median lifespans[1]			ave lifespan change due to DR[2]
		Trial 1	Trial 2	Trial 3	
Dahomey	0.1	17.1	18	ND	
	0.5	**73**	**73.5**	**69.1**	
	1	65	66.5	59.5	31%
	1.5	63	64	48	
	2	*57*	*55*	*52.5*	
yw	0.1	ND	ND	ND	
	0.5	**69**	ND	**73.5**	
	1	*61*	ND	*66.5*	12%
	1.5	ND	ND	ND	
	2	61	ND	48	
w1118	0.1	22	ND	ND	
	0.5	53.1	ND	ND	
	1	**57**	ND	ND	36%
	1.5	48.4	ND	ND	
	2	*42*	ND	ND	
CantonS	0.1	15	18	ND	
	0.5	53.1	**57**[3]	**50**	
	1	**57**	**59.5**	48	28%
	1.5	55.1	52.5	*38.5*	
	2	*46.1*	*45.5*	*38.5*	
OregonR	0.1	17	22	ND	
	0.5	**53.1**	45.5	**66.5**	
	1	**53.1**	**52.5**	59.5	15%
	1.5	*50.9*	*48*	*55*	
	2	42	45.5	52.5	
wDahomey	0.1	18.5	ND	ND	
	0.5	**73**	ND	ND	
	1	57	ND	ND	66%
	1.5	57	ND	ND	
	2	*44.1*	ND	ND	

[1]bold numbers denote the greatest median lifespans and italicised numbers the shortest median lifespans, within the DR food range for that strain in that trial.
[2]For all DR ranges for each strain, the longest-lived condition was significantly different from the shortest-lived condition; percentages are derived from the average lifespan difference due to DR.
[3]In cases where there was no significant difference between two food types for the longest or shortest-lived condition, two numbers are in bold or italicised.
doi:10.1371/journal.pone.0004067.t001

Upon testing our wild-type strains for *Wolbachia* we found all except w1118 and OregonR were infected (Figure 4a). Therefore, because all strains exhibited a DR response, *Wolbachia* infection *per se* can not account for the full effect of nutrition on lifespan. To test if tetracycline-treatment could eliminate the DR response by other means, we selected three lines for treatment (Dahomey, CantonS and OregonR). After two generations on tetracycline-containing food, flies were subsequently maintained on normal food to recover for at least five generations. PCR testing revealed that the treatment was effective as both Dahomey and CantonS were cleared of *Wolbachia* (Figure 4b). When subjected to different food concentrations, all three tetracycline-treated lines retained their DR response (Figure 4c). In the trial shown, the lifespan peak for all three strains was at 1× food and fecundity increased to 2× food. While this was qualitatively different from that seen in the previous trials with non-tetracycline-treated flies, a further trial with these lines after an additional five generations on normal food, revealed more similar data to that shown in Figure 3 (data not shown). Thus, tetracycline-treatment may produce a transitory alteration in the way flies respond to food, but its effects can not account for the DR response.

Discussion

Intermittent feeding did not extend fly lifespan but does not rule out DR in flies

There are several different ways to restrict the access of animals to nutrition and thus extend lifespan by DR [27]. For flies, dilution of the concentration of yeast in a diet that is provided in excess, has proven to be practical and effective [12,28]. However, food dilution methods are unique to the invertebrates and in mammalian studies, periodic access to food is used. One such

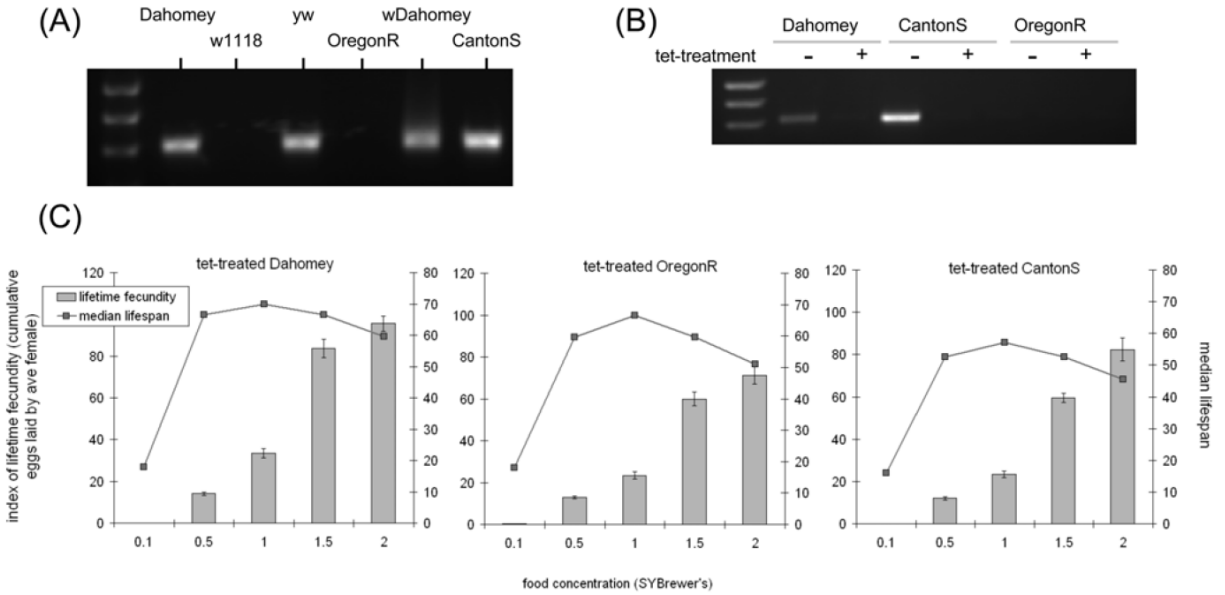

Figure 4. Tetracycline treatment does not eliminate the DR response. (A) Gel showing diagnostic PCR for the presence of the intracellular bacterium *Wolbachia*; (B) three strains were selected from the set of wild types for treatment with tetracycline, which was sufficient to clear *Wolbachia* if present. (C) Each of the three strains was then allowed at least five generations to recover on non-tetracycline-containing food before being assayed for lifespan and fecundity on different concentrations of food. Each of the three strains still exhibited a DR response after tetracycline treatment. Bars: index of lifetime fecundity±standard error of the mean; connected points: median lifespan in days. Data shown are from one of two trials in which all lifespans were run simultaneously.
doi:10.1371/journal.pone.0004067.g004

protocol provides animals with a measured amount of food that is completely consumed before the next meal. While effective for extending rodent lifespan [7], it has been unsuccessful when used on flies [29,30]. An alternative technique is EOD feeding, which extends rodent lifespan by alternating periods of access to excess food with periods of starvation. Importantly, the mice subjected to this regime increased their feeding behaviour such that they consumed nearly the same quantity of nutrients as controls. Thus, regular periods without food maybe just as important as reducing nutrient intake for extending rodent lifespan [16]. In contrast, this protocol has had little or no success when adapted for flies [18,19]. In this study, we also found no extension of life using a similar protocol on *Drosophila* (Figure 1). Thus, our results support the previous invertebrate data and could be used to argue that periods of starvation cannot extend the lifespan of flies [30,31] or that the mechanism by which DR extends lifespan is different between flies and mammals. While both of these explanations are possible, the fact that lifespan was not shortened by the more severe of our restriction treatments means we are unable to determine how much nutrient intake may have been reduced, or exactly what other periods of starvation could be protective for lifespan in our flies. While a more extensive range of starvation periods would be revealing, other factors such as the time of day at which food is removed may also be important since feeding behaviour is controlled by the circadian rhythm [32]. Thus, it is easy to implement an inappropriate methodology when attempting to DR flies in this way and the absence of a positive result does not rule out the possibility of observing a positive effect if protocols were optimised.

If nutrient restriction is the critical factor in these DR experiments then intermittent feeding protocols that use different dietary compositions would also be expected to vary lifespan outcomes in different ways. Figure 5 illustrates how this is possible. When given increasing doses of a relatively concentrated diet (orange line), lifespan would increase as malnutrition lessens to a peak at an intermediate level of food availability. As food availability is increased beyond this point, lifespan decreases via the DR response. At some point, no additional increase in food availability will further shorten lifespan as the organism will reach its limit to ingest more food ('point of satiety' and beyond). If, however, the concentration of the food being provided is low enough (represented by the 'dilute' and 'very dilute' diets in Figure 5), lifespan will increase to a plateau whose onset occurs at the point that the organism's food intake limit is reached. If these dilute food types are used in an intermittent feeding protocol, it would be impossible to find an intermediate level of food exposure which increases lifespan, falsely giving the impression that DR does not exist. It is possible that this can explain why some studies have been published that did not find a DR response (eg [33–35]). As mentioned above, food dilution has proven to be the most successful intervention to implement DR in flies [11]. The connection between this intervention, where the food remains in excess, and intermittent feeding can be found by taking the lifespan values at any one level of food availability above the point of satiety in the left panel of Figure 5. A cross-section of these values is shown in the right panel of Figure 5; this represents the standard DR effect in flies (eg Figure 2). It should be noted that in reality, this illustration is somewhat simplistic in that the lifespan-sensitive nutrients represented on the x-axis are unlikely to be accurately represented by the term 'food availability'. Furthermore, nutrient composition variations are likely to alter the point of onset of satiety, which in turn changes the onset of the lifespan plateau. Thus, although further work on diet composition, feeding intervals and measured food availability may uncover an

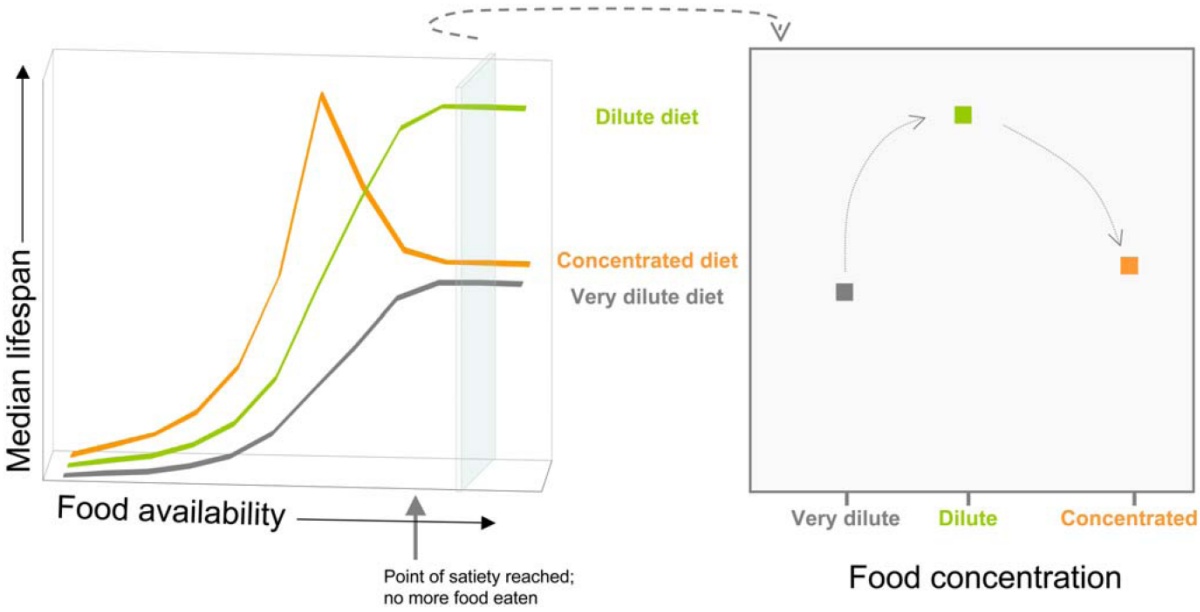

Figure 5. Model of the relationship between lifespan and DR protocols that reduce access to food either by intermittent exposure (left panel) or nutrient dilution (right panel). These demonstrate how the composition of food used for intermittent feeding protocols could lead to the false conclusion that DR does not exist for an organism. Three different diets are shown that vary in a given nutrient concentration from 'very dilute' to 'concentrated'. In this example, increasing access to the concentrated diet causes lifespan to rise to a peak (DR) beyond which lifespan decreases. At some point (marked here as the 'point of satiety') the animal will no longer be able to eat any more food, meaning the nutrition level it experiences is capped and no further increase in availability will further decrease lifespan. For the dilute and very dilute diets, the point of satiety is reached before the level of nutrients ingested has a chance to cause lifespan to reduce. Thus, there is no lifespan increase for any intermediate level of food restriction, making it look like the organism does not exhibit a DR response. For flies, these problems can be avoided by assaying lifespan in the presence of excess food that is diluted to differing extents. The relationship of this situation to DR by intermittent feeding is represented by taking a cross-section through the graph on the left. The plot on the right shows the type of data presented herein and for other invertebrate studies.
doi:10.1371/journal.pone.0004067.g005

alternative intermittent feeding regime suitable for flies, it is likely to be a labour intensive process that may not provide any more information about DR than dietary dilution.

DR in Drosophila does not appear to be a laboratory artefact

For ease of handling and to extend generation times, fly stocks in the laboratory are often kept in small numbers, under relatively poor nutrient conditions and at low temperatures. Over time, these factors are likely to exert selective pressures that could influence lifespan. Importantly, when transferring stocks to fresh food for maintenance, it is relatively easy to select for early age of reproduction, which is known to reduce adult lifespan [36]. That this happens in the laboratory has been demonstrated by comparing the lifespans of flies maintained for years in the laboratory under normal stock-handling conditions with others selected for early or late reproduction as well as others freshly caught from the wild [37]. This study showed that the laboratory stocks were as short lived as those selected for early reproduction, while the wild-caught lines had a much longer lifespan, similar to flies selected for late reproduction and were much longer lived. In our laboratory, we have maintained a wild-type outbred stock (Dahomey) since 1970 in large population cages with overlapping generations. When compared with other laboratory wild-type strains that we have maintained using routine stock handling techniques, we found that Dahomey demonstrated the capacity for both the longest lifespan and the greatest lifetime egg laying output (Figure 6). Thus, maintenance of flies using large population cages with overlapping generations appears to preserve the life history characteristics of wild-flies for long periods of time. This is in agreement with previous work that demonstrated this fact for flies maintained in the laboratory during a three year period [38].

Importantly, despite the differences between strains in their selection histories, all exhibited a DR response (Figure 3). Recently, it has been proposed from work with mice that lifespan extension by DR could simply be an artefact of laboratory domestication because a wild-caught strain was reported whose longevity was not increased in response to a typical DR regime [15]. In contrast, a recent study of several wild-derived strains of *C. elegans* showed that all exhibited a DR response [39]. Although we have not directly tested DR using flies recently caught from the wild, our study indicates that they would exhibit a DR response because of the strong effect seen with Dahomey (Figure 3 and Figure 4). It should be noted that the invertebrate studies were conducted using a DR technique that deprived worms of bacteria, while the rodent study used a food restriction protocol with only one level of limitation. Thus, as explained above, diet design and an incomplete range of food concentrations could be important factors in explaining why the DR effect was apparently absent from wild mice [15].

DR in Drosophila is not sensitive to tetracycline treatment, but varies with diet quality and genotype

We show here that DR is not sensitive to infection with the bacterium *Wolbachia*, or indeed any other tetracycline-sensitive infection that may be present in flies (Figure 4). Interestingly, the levels of fecundity at a given food concentration differed after

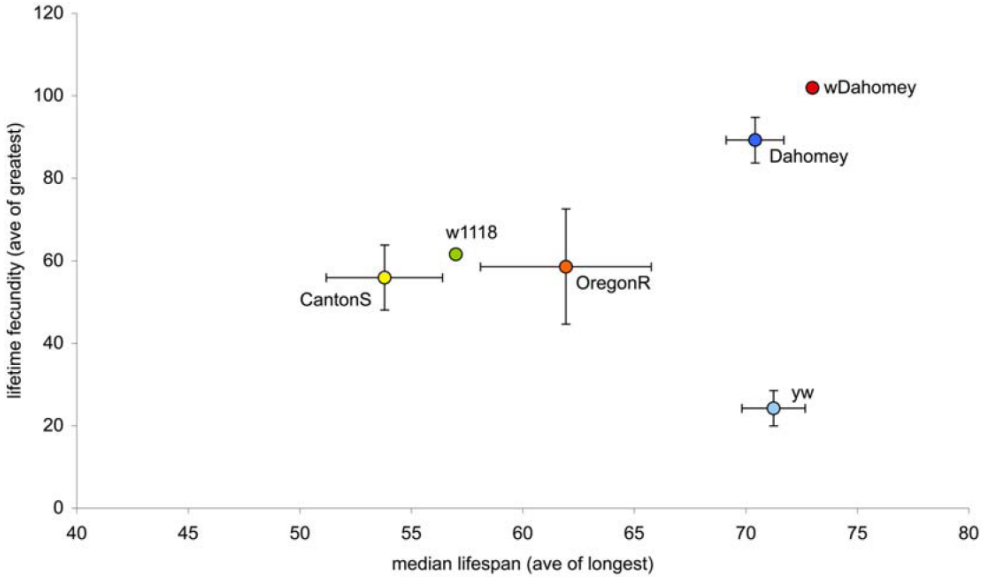

Figure 6. The Dahomey genetic background is capable of the longest lifespan and greatest reproductive output of the wild-type strains tested. For median lifespan, the data are the averages from the longest lived conditions for each strain. For lifetime fecundity they are the average of the condition producing the greatest lifetime reproduction. It should be noted that the conditions under which these occur is different for the two traits, as predicted by the expectations of DR, and that they may be different for each different strain. Data from n independent repeats, where n = 5 for Dahomey, CantonS and OregonR; n = 2 for yw, and; n = 1 for w1118 and wDahomey.
doi:10.1371/journal.pone.0004067.g006

tetracycline treatment (compare Figure 3 with Figure 4c). This indicates that some tetracycline-sensitive microbes carried by some flies might be involved in the control of fecundity. However, further data would be required to validate this observation since a subsequent trial with the tetracycline-treated flies saw fecundity levels return to those previously observed (as for Figure 3; data not shown). Together, these data extend previous work we have performed to optimise a DR protocol to avoid lifespan variations from non-nutrient dependent effects [12]. From this work, we have sought to generate a standardised DR protocol to aid studies into the mechanisms of DR. However, we here report that the food concentration to yield the longest lifespan in Dahomey was at 0.5×, which is less than the 1× reported in [12]. This demonstrates an inherent problem with using a natural ingredient like yeast whose nutritional content varies seasonally due to production methodology and the quality of its feedstock. In doing so, it also highlights the need for a standardised synthetic defined medium to replace yeast-based diets to study the details of how lifespan varies with food composition. Interestingly, not all strains exhibited a lifespan peak at the same food concentration as Dahomey (Figure 3). It is already known that genotype can affect the interaction between lifespan and food [27,40–42] and could indicate the breadth of the DR effect on fly health. One interesting possibility from these data is that if flies of different genotypes die from different pathologies, DR has the ability to delay the onset of each of these causes of death, which agrees with data from rodent studies [7,43]. Future work on the exact molecular mechanisms of DR via interactions with different genotypes on precise dietary manipulations will be key to exploring this further.

Materials and Methods

Fly stocks and maintenance

Dahomey: This strain has been in the laboratory since 1970, having been collected in West Africa in what is now the Republic of Benin. Four population cages (dimensions: 20 cm H×21 cm W×30 cm D) have been maintained in parallel at 25°C on a 12-hour light/dark cycle. At all times, 12 bottles of food are in each cage, being replaced gradually. Each week, three half-pint bottles containing 70 ml of food (1× SY) are supplied to each cage and the three oldest bottles removed.

wDahomey was generated by backcrossing the white gene from w1118 into the Dahomey genetic background. It has since been maintained in one large population cage with a feeding regime as described above for Dahomey.

w1118, yw, OregonR and CantonS have been maintained in the lab for many years under a variety of conditions. Generally, this involves transferring each new generation to a fresh set of several half-pint bottles or vials of food. These are usually kept at 18°C to extend each generation's lifecycle and are fed either 1× SY food or a cornmeal-based diet (see below).

Media

The SY food reported here is the same as SYBrewer's in [12]. Standard (1×) contains per litre: 100 g autolysed Brewer's Yeast (MP Biomedicals, Solon, OH), 100 g sucrose (Tate & Lyle sugars, London, UK), 15 g agar (Sigma, Dorset, UK), 3 ml propionic acid (Sigma, Dorset, UK), 30 ml Nipagin M solution (100 g/l methyl 4-hydroxybenzoate in 95% ethanol) (Clariant UK Ltd, Pontypridd, UK), distilled water to 1 l. Cornmeal-based diet used in stock keeping contains: 60 g cornmeal (organic polenta; B.T.P. Drewitt, London, UK), 20 g autolysed Brewer's yeast, 85 g sucrose, 10 g agar, 25 ml Nipagin M and 1 l distilled water.

Tetracycline treatment was carried out by the addition of 25 μg/ml tetracycline to 1× SY food for two generations.

For stock maintenance, food was cooked in a 60 l Joni Multimix food preparation kettle (Joni Foodline, Munkebo, Denmark), while experimental food was prepared on a gas hob as described in [12].

Lifespan and fecundity assays

Flies were reared at a standard density for at least two generations before being used for lifespan experiments as previously described [12,44]. All experiments were performed with female flies that were allowed 48 h to mate after emerging as adults. On the second day of adult life, flies were lightly anaesthetized with CO_2, sorted and counted at 10 per vial. The minimum number of flies per condition was 100. For the intermittent feeding experiment, five replicate 1 l cages, each containing 100 flies was used for each condition. These cages have two side-arm inlets that can each accommodate a food vial. The periods of starvation were initiated at 10:00 (lights on), whereupon the food vial (2× SYBrewer's) was replaced with an empty vial. In all cages at all times, flies had constant access to a vial containing water that was plugged with wet cotton wool. This was housed in the side-arm not containing the food vial. In all cases, flies were transferred to fresh food at least three times a week, at which point deaths were scored.

For fecundity measurements, eggs were counted after the flies had been in the vials for between 18 and 24 h. Generally, these counts were performed once a week for the first six to seven weeks of adult life. Importantly, the first egg count was only conducted after at least four days exposure to the new food in order to allow time to adjust to the new nutritional conditions.

PCR detection of Wolbachia infection

PCR for detection of Wolbachia was performed using primers wsp81F and wsp691R (kind gift from G. D. D. Hurst) as described in [45]. In each case, a sample of flies form the experimental generation was used for PCR testing.

Data analyses

Lifespans were recorded and analysed using spreadsheets created in-house in Excel. Comparisons using the Wilcoxon rank sum test were performed in R, v2.5.1 [46].

Author Contributions

Conceived and designed the experiments: RCG RW TMB LP MDWP. Performed the experiments: RCG RW TMB MDWP. Analyzed the data: MDWP. Contributed reagents/materials/analysis tools: RCG RW TMB MDWP. Wrote the paper: LP MDWP.

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assays in eGFP storage buffer containing 10 mM EDTA. We performed all our experiments in standard actin-myosin ATPase assay buffer, after 100-fold dilution of GFP storage buffer and many wash steps (nominal EDTA–free). Furthermore, under this experimental condition, the effect of GFP is concentration-dependent. Our results in GFP-expressing cells also demonstrate a dissociation between the two cell processes, that is, normal calcium transients that are not translated into cell contraction, pointing to a GFP-induced impairment of electro-mechanical coupling. This has been confirmed by Nishimura and colleagues[3]. We do not intend our results to detract from the use of GFP fusion proteins, especially GFP-myosin, which restricts localization of GFP to a cellular compartment, thereby limiting its toxicity[6,7]. However, our results suggest that free GFP in excitable cells should be used with approaches that allow control of its expression.

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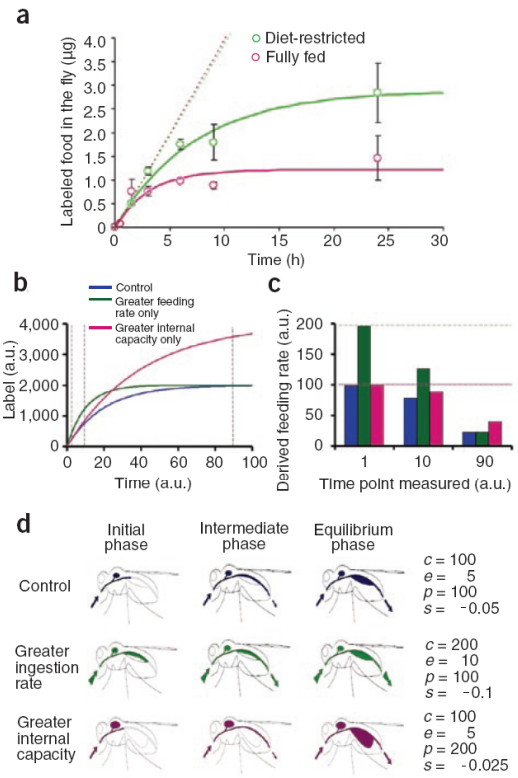

Figure 1 | Characteristics of labeled food accumulation in flies. (**a**) The amount of labeled food present in diet-restricted and fully fed flies at different times after transfer from unlabeled food to food labeled with blue food dye. Solid lines, measured dye accumulation; dashed lines, label accumulation profile that would occur if feeding rates were the only factor governing label accumulation. Error bars, s.e.m.; $n = 6$ for each time point. (**b**) Modeled dynamics of accumulation of dye with time for flies assigned arbitrary values of feeding rate, c; internal label capacity, p; and rate of label removal, e. (**c**) Feeding rates for the three conditions modeled in **b**, derived by measuring the label accumulated at the indicated arbitrary times. If sampled during the 'initial phase' ($t = 1$ arbitrary unit), the observed feeding rate reflects the real feeding rate (dashed lines). During the 'intermediate phase' ($t = 10$ arbitrary units), the apparent feeding rate is lower than the real rate and more so in the fly with the higher feeding rate. In the 'equilibrium phase' ($t = 90$ arbitrary units) the apparent feeding rate falsely gives the impression that flies represented in magenta have a higher feeding rate than those represented in blue or green. At this point, the measurement only reflects the internal capacity of the fly for the label. (**d**) A pictorial representation of what may be occurring in the fly for each condition. Fraction of label turnover, s.

Pitfalls of measuring feeding rate in the fruit fly *Drosophila melanogaster*

To the editor: Dietary restriction, a reduction of food intake without malnutrition, extends lifespan in many organisms, including *Drosophila melanogaster*. In *Drosophila*, dietary restriction can be implemented by food dilution[1]. Flies could compensate for reduced nutrient content of food by increasing their feeding rate. Food intake is difficult to measure in *Drosophila* owing to their small size. A recent study used radioactively labeled food to estimate *Drosophila* feeding rate and reported compensation for food dilution[2]. The authors assumed that the amount of radioactive label in flies depended only upon rate of ingestion. However, retention time of the food can also vary. Without measurement of this variable, genuine differences in feeding rate could be undetected or spurious differences in feeding rate could be detected. We illustrate this here using a mathematical model and an experiment using food containing a non-absorbed food dye[3].

We measured dynamics of dye accumulation in flies after transfer to labeled food (**Fig. 1a**). Initially, diet-restricted and fully fed flies accumulated label at similar rates. However, rate of accumulation declined faster and label reached lower equilibrium levels in fully fed than in diet-restricted flies. Had dye accumulation reflected only feeding rate, these results would imply that up to 30 min there was no feeding rate difference between groups but that by 3 h of feeding there was an approximately 1.5-fold higher feeding rate in the diet-restricted flies. We therefore considered the possibility of a difference in retention time for the food. We found that diet-restricted flies had 45% larger crop size than fully

fed flies ($P < 0.0001$, Wilcoxon rank sum test; **Supplementary Methods** online). When we exposed flies briefly to dye-labeled food, we found that the dye took less than 50 min to start appearing in feces. Thus, by 30 min, the amount of dye accumulated in the fly reflected feeding rate alone, while after 50 it reflected the rate of label ingestion, the rate of egestion and the gut capacity. Our measurements of crop size showed that the latter was increased by dietary restriction[4]. The use of radioactive labels[2] involves further potential confounding processes than those for a non-absorbed dye because the amount of isotope present will also depend upon the capacity of the body for the labeled element[2,5].

Using data from Geer *et al.*[5] for ^{14}C-choline labeled food accumulation by *Drosophila* (**Supplementary Fig. 1** and **Supplementary Methods** online), we generated a model:

$$m(t) = -\left(\frac{c}{s}\right) \times [1 - \exp^{(s \times t)}]$$

where $m(t)$ is the amount of label in the fly at time t; c is the feeding rate; and s is the fraction of labeled material removed from the fly (rate of label removal divided by the internal label capacity of the fly). We assigned arbitrary values to these parameters and observed their effect on label accumulation (**Fig. 1b–d**). The accumulation profile (**Fig. 1b**) consists of an 'initial' phase when label is taken in and not egested, an 'intermediate' phase where label ingestion rate exceeds egestion rate, and an 'equilibrium' phase when label egestion and ingestion rates are equal. The amount of label in the fly gives a reliable estimate of feeding rate only during the 'initial' phase. During the 'intermediate' phase, the amount of label in the fly will underestimate the extent of a genuine difference in feeding rate (**Fig. 1b–d**) and will fail to detect the difference once 'equilibrium' is reached. For a fly with a greater internal capacity, the amount of dye present will overestimate feeding rate relative to controls once egestion has started ('intermediate' phase), to an extent that reaches a maximum at the 'equilibrium' phase (**Fig. 1c**).

Fitting this model to the data presented in **Figure 1a**, diet-restricted and fully fed flies consumed food at equal rates, but fully fed flies turned over 32% of their gut capacity per hour, whereas diet-restricted flies turned over only 14%. At equilibrium, the absolute amount of material egested must equal the amount eaten, and therefore diet-restricted flies have an approximately twofold larger gut capacity for labeled food than do fully fed flies. Thus the conclusion that fruit flies compensate for dietary restriction by increasing their feeding rate[2] was inaccurate because of inappropriate use of the method. As an alternative for longer-term measurements, we have developed an assay that, when appropriately calibrated, offers a more accurate measurement of food intake[6].

Note: Supplementary information is available on the Nature Methods website.

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Carvalho *et al.* reply: In their Correspondence, Wong *et al.*[1] argue that dye labeling is not a satisfactory technique to address the issue of compensatory feeding and try to generalize this inadequacy to all food labeling methods. While some valid points are raised, others are off the mark.

As the authors point out, long-term measurements made with food labels reflect not only ingestion, but also internal capacity for the label and elimination rates. Capacity is a major limitation of non-absorbable dyes but not of ^{32}P or ^{14}C radiolabeling. Whereas dye measurements quickly plateau[1], ^{32}P and ^{14}C levels accumulate near-linearly for several days[2,3], indicating that internal capacity is not rate-limiting over 24 h, the time point used in our study[2]. In contrast, as emphasized in our report, the absorbable nature of isotopes precludes us from discerning whether compensation takes place at the level of intake or absorption. Using the capillary feeder (CAFE), a direct, real-time assay of ingestion in undisturbed animals involving no food labels, it has been recently demonstrated that flies can sense nutrient variation and adapt their intake accordingly[4], supporting the behavioral over the metabolic mechanism for compensatory feeding. As for elimination, the fact that diluted media stimulate excretion[5] suggests that compensatory feeding may be even more dramatic than our results indicated. However, this does not directly address isotope turnover. It will be interesting to dissect this issue using methodology developed since the publication of our report[6].

Radiolabeling and the CAFE are currently the most reliable and sensitive assays of feeding behavior in adult *Drosophila melanogaster*. With the advent of these methods, it is, in our opinion, no longer justifiable to infer feeding rate from surrogate assays such as egg laying or indirect behavioral observations[7].

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Quantification of Food Intake in *Drosophila*

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Abstract

Measurement of food intake in the fruit fly *Drosophila melanogaster* is often necessary for studies of behaviour, nutrition and drug administration. There is no reliable and agreed method for measuring food intake of flies in undisturbed, steady state, and normal culture conditions. We report such a method, based on measurement of feeding frequency by proboscis-extension, validated by short-term measurements of food dye intake. We used the method to demonstrate that (a) female flies feed more frequently than males, (b) flies feed more often when housed in larger groups and (c) fly feeding varies at different times of the day. We also show that alterations in food intake are not induced by dietary restriction or by a null mutation of the fly insulin receptor substrate *chico*. In contrast, mutation of *takeout* increases food intake by increasing feeding frequency while mutation of ovo^D increases food intake by increasing the volume of food consumed per proboscis-extension. This approach provides a practical and reliable method for quantification of food intake in *Drosophila* under normal, undisturbed culture conditions.

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Introduction

The fruit fly *Drosophila melanogaster* is a key model organism for discovery of evolutionarily conserved biological mechanisms, which include the control of nutrient sensing [1,2], feeding [3–5] and ageing [6,7]. Reliable methods for measuring food intake of *Drosophila* are therefore often needed. However, quantification of food consumption in the fly poses challenges. In mammals, food ingestion can be directly quantified by weighing the food before and after feeding has taken place. However, flies consume volumes of food that are too low to weigh accurately, and feed by extension of their proboscis into the food medium, prohibiting direct observation of the volume of food ingested. One method has overcome this problem by measuring the food consumed in liquid form in a capillary feeder (CAFE) [8]. However, despite being effective for quantifying intake, CAFE feeding substantially reduces both the egg-laying and lifespan compared to those seen in flies provided with food in the usual agar-gelled medium [7,9]. This may be because in nature *Drosophila* feed on microorganisms, particularly yeast, on the surface of fruit [10,11], and thus feeding on a liquid diet from a capillary may not reflect their natural feeding environment.

To overcome the problems of measuring food intake when flies feed on gelled media, several studies have made indirect measures of food uptake after marking the food, either with a visible dye [12–14] or with radioactively-labelled nutrients [15–17]. However, such 'tracer' methods have limitations and can even give misleading results. Transferring flies to labelled food creates a disturbance that could change the volume of food ingested per proboscis-extension (ingestion ratio) and/or the frequency of proboscis-extension (feeding frequency), and therefore measurements immediately after transfer may not be an accurate reflection of food consumed during undisturbed conditions. Furthermore, because tracer methods rely on measuring only the volume of label present in the fly, the results can be influenced by factors other than feeding, and substantial differences in either the ingestion ratio or feeding frequency can be over-looked [18]. For instance, if the internal capacity of the flies for the label is increased by the experimental treatment, with no alteration in feeding, then with increasing times of exposure to the labelled food, the group with the higher internal capacity will give the spurious appearance of having a higher food intake. This problem can occur with dietary restriction in *Drosophila*, which increases the capacity of the crop [18]. In addition, if flies differ in food intake but not in internal capacity for the food tracer, then once steady state is reached with rate of egestion of the label equalling the rate of intake, the amount of label present in the two groups of flies will be the same, despite their difference in food intake [18]. For the amount of label in the fly to reflect feeding, measurements must therefore be confined to the time period before label egestion commences, about 40 minutes in *Drosophila*, a time period during which disturbance of the flies affects their feeding behaviour. There is thus a requirement for a method of measuring feeding in undisturbed conditions.

Previously, we have reported that direct observations of fly proboscis-extension onto the food surface [19] can indicate food intake. This assay offers three advantages over the methods mentioned above: 1) repeated assays can be performed with the same flies through time because no flies are sacrificed for measurements, particularly valuable in the context of work on ageing; 2) the observations can be made on flies housed on standard laboratory food, and could be extended to other culture conditions; 3) food intake can be measured during undisturbed conditions once the proboscis-extension observations are calibrated by measures of short-term dye-accumulation, to determine the volume of food ingested per proboscis-extension.

In this study we tested the accuracy of the method, by measuring the volume of food ingested using a dye food label in parallel with observing the number of proboscis-extensions. We compared flies that had either known or suspected differences in food intake, such as males versus females, flies subjected to dietary restriction [20] and *chico*^{1} [21], *takeout*^{1} [22] and *ovo*^{D1} [23] mutant flies relative to their controls. Additionally, we also checked that the ingestion ratio did not alter with age, by performing the combined assay on flies over various days of a lifespan.

Dietary restriction (DR) in *Drosophila* is often achieved by dilution of the food medium, and complete records of food intake are needed to determine if flies compensate for the reduced nutritional content of food by increasing the total amount of food they consume. Measurement of food intake is also needed to determine if other interventions, such as sensory perception of food [24], or reduced insulin/insulin-like growth factor signalling (IIS) [6,25] extend lifespan by reducing nutritional intake and hence act by inducing a state of DR. We tested this possibility by measuring the food intake of flies carrying a mutant for the IIS gene *chico*, which extends the lifespan of *Drosophila* [21].

Results

Establishing a relationship between proboscis-extension and total volume of food eaten

In nature and in the laboratory, fruit flies feed on the food surface, by extending their proboscis into contact with the food and drawing it in. The amount of proboscis-extension onto the food surface was measured by making periodic observations of groups of flies. The number of observations of proboscis-extension was then expressed as a proportion of the total number of observations [19]. Short-term food consumption was quantified by transferring flies onto food labelled with a non-toxic, non-absorbed blue dye. The amount of blue food present in the fly was quantified using spectrophotometry [12]. The assay period was confined to the 30 minutes after transfer, because the dye is egested shortly after this length of time [18]. Thus a 30-minute exposure period to blue dyed food ensured that all dyed food eaten during the assay is retained in the fly gut and none was lost by egestion.

To compare proboscis-extension measurement against dye ingested, we performed the two assays described above on the same cohort of flies. Initially, groups of 5, 7-day-old mated female flies were allowed to feed for 30 minutes on food labelled with blue dye [12] while we simultaneously observed the proportion of time they spent with the proboscis extended [19]. Flies were then sampled, and the amount of blue food they ingested quantified. We then plotted the level of blue food measured in the group against the proportion of proboscis-extensions observed in that group (**Figure 1a**). We found a strong positive linear relationship between the Volume of blue food found in the fly and the proportion of feeding events Observed (V/O) ($P<0.0001$, linear mixed effect model, LMEM). The gradient of this relationship represents the ingestion ratio of the flies, as it describes the volume of blue accumulated per proboscis-extension. To test for non-linearity (for example, saturation or acceleration in the V/O relationship), we added a quadratic term to the statistical model. The quadratic term was not significant ($P=0.62$), indicating the V/O relationship is indeed linear over the timespan we measured (**Table 1**). The linear relationship demonstrated that the proboscis-extension method is an accurate indicator of food intake in female *Drosophila* under these conditions.

Next, we tested whether the sexes differed in ingestion ratio (gradient of the V/O relationship) by repeating the combined assay with 7-day-old mated males and females (**Figure 1b**). The ingestion ratio was constant in males and in females, as both were found to have a significant V/O relationship ($P<0.0001$, LMEM). The gradients of these relationships were not found to be significantly different ($P=0.9871$), indicating that the ingestion ratio did not differ between the sexes. However, the intercept of the male relationship was significantly lower than that for females ($P<0.001$) and suggested males across all observations contained a lowered basal level of blue dye content than in females (**Table 1**). This could be due to differences in body size and/or body composition (e.g., proportions fat, muscles and reproductive tissues). As in the previous analysis, the quadratic term was not significant ($P=0.54$), indicating that a linear V/O relationship exists. In spite of the sexes sharing the same ingestion ratio, females were found to have fed more than males over the 30-minute period because they spent a greater proportion of time with the proboscis extended (2.8-fold more on average) than males ($P<0.0001$, generalised linear model, GLM). This suggested it is possible for flies to increase their food intake by feeding at a greater frequency rather than by consuming in greater volume, and it is possible to detect such differences in food intake.

We then extended the use of this method to examine the effect of other factors that could determine the physiology and behaviour of feeding flies. The nutritional environment may be such a factor, and is particularly important in the context of DR experiments where dietary dilution is employed to restrict access to nutrients. We therefore performed the combined assay with 7-day-old mated females that were fed either DR or full fed control diet [7] (**Figure 1c**). Flies on differing yeast concentrations did not alter the ingestion ratio, because no significant difference in V/O relationship was found ($P<0.0001$, linear regression model), with no significant differences in the gradient and intercept of this relationship between the two different diet regimes ($P=0.447$, respectively, $P=0.304$: **Table 1**). Flies on the DR diet were also found not to compensate for the reduced nutrient availability by feeding more often, because the proportion of proboscis-extensions between DR fed and full fed flies during the 30-minute period of the combined assay were not different either ($P=0.3693$, GLM).

Finally, we also tested whether the ingestion ratio or feeding frequency were altered by genetic mutations known or suspected to affect feeding. The first mutation, *chico*^{1}, is a null mutation in the single fly insulin receptor substrate in the insulin/insulin-like growth factor-1 signalling (IIS) pathway, a pathway suggested to affect foraging and feeding in larvae [26]. We performed the combined feeding assay on 7-day-old mated female heterozygotes of *chico*^{1} and their genetic control (Dahomey) (**Figure 1d**). The ingestion ratio did not differ between *chico*^{1} heterozygotes and their controls, because a significant V/O relationship exists ($P<0.0001$, LMEM), with no significant differences in the gradient or intercept between *chico*^{1} heterozygotes and control flies ($P=0.3177$, respectively, $P=0.3947$, **Table 1**). *chico*^{1} heterozygous flies and their controls had the same food intake, because the proportion of proboscis-extensions between the cohorts during the 30-minute period of the combined assay were also not significantly different ($P=0.0831$, GLM).

The second mutation, *takeout*^{1}, is in a gene reported to regulate the circadian rhythm and to increase food intake prior to starvation in *Drosophila* [22]. We performed the combined feeding assay on 7-day-old mated *takeout*^{1} flies and their genetic control (Canton-S) (**Figure 1e**). The ingestion ratio did not differ between *takeout*^{1} flies and controls, because a significant V/O relationship existed ($P<0.0001$, linear regression model) with gradient and intercept not significantly different between the two genotypes ($P=0.5931$, respectively $P=0.0549$: **Table 1**). This suggested that

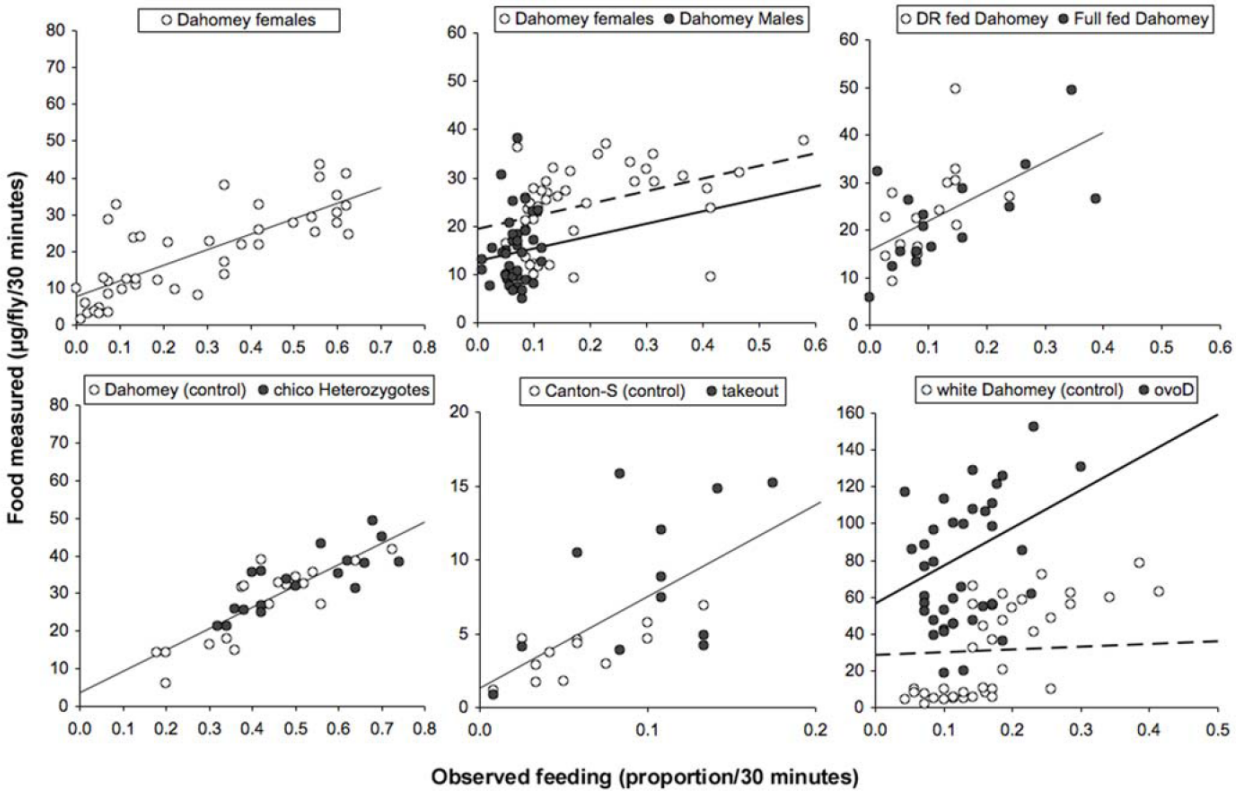

Figure 1. Measurements of blue label uptake after 30 minutes of feeding and the proportion of feeding events observed during this period, where one circle represents one vial containing 5 flies. Trend lines represent the relationship between the volume of food ingested and the observed proportion of flies feeding (V/O) described in **Table 1**. Dashed lines represent open circles. All flies were female unless stated, were 7 days old and were allowed to mate for 48 hours after eclosion (NF = the number of flies per condition, NV = the number of vials per condition). (**a**) A linear (V/O) relationship existed in mated Dahomey females (NF = 210, NV = 42). (**b**) The V/O relationships of mated Dahomey males and females did not differ significantly, although females were found to have fed at a greater frequency than males during the 30 minutes (NF = 200, NV = 40). The gradient for males did not differ significantly from that for females but had a lower intercept. (**c**) DR fed and full fed Dahomey females shared the same V/O relationship and no difference in feeding between dietary conditions was found with the combined assay (NF = 75, NV = 15). (**d**) The V/O relationship was the same in *chico*^{1} heterozygotes and in the Dahomey control. No difference in feeding between genotypes was found with the combined assay (NF = 90, NV = 18). (**e**) The V/O relationship was the same in *takeout* ^{1} and in Canton-S females, even though *takeout* ^{1} flies were found to feed at a higher frequency than Canton-S controls (NF = 60, NV = 12). (**f**) Both *ovo*^{D1} and *white*^{Dahomey} females had a positive V/O relationship, but *ovo*^{D1} flies had a significantly greater gradient and intercept, and therefore increased the volume of food ingested per proboscis-extension more quickly than *white*^{Dahomey} females (NF = 200, NV = 40).
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the ingestion ratios in both *takeout*^{1} flies and controls were similar. However, *takeout*^{1} flies fed more than controls, because they spent 1.6-fold more time with their proboscis extended on the food than did Canton-S flies ($P<0.05$, GLM). The flies thus elevated their nutrient-intake by feeding at a greater frequency, rather than by increasing the volume of intake per proboscis-extension.

The final mutant studied, *ovo*^{D1}, causes female sterility and has been reported to induce a reduced feeding frequency [23]. We performed the combined assay with 7-day-old, mated, mutant females and their genetic control (*white*^{Dahomey}) (**Figure 1f**). A significant V/O relationship was found for both cohorts ($P<0.0001$, LMEM); however, the gradient and the intercept for the relationship differed between the two genotypes ($P<0.0001$ and $P<0.001$, respectively). The V/O gradient for *ovo*^{D1} was steeper (205.52 versus 14.46 in *white*^{Dahomey}) and the intercept greater (56.40 versus 28.65 in *white*^{Dahomey}) than for *white*^{Dahomey} controls (**Table 1**). *ovo*^{D1} females thus had ingested a greater volume of food per proboscis-extension compared to *white*^{Dahomey} controls (accumulated blue dye faster with each proboscis-extension), as well as a greater basal level of blue dye. However, no significant difference in the proportion of time spent feeding between *ovo*^{D1} females and *white*^{Dahomey} controls was recorded ($P=0.6289$, GLM). This indicated that *ovo*^{D1} flies elevated their received nutrition by increasing the volume of intake per proboscis-extension rather than by feeding at a greater frequency.

We also analysed the effect of age upon the ingestion ratio. Dahomey females were subjected to the combined blue dye and proboscis-extension assay at 4 different ages (day 7, 21, 35 and 50: **Figure 2**). The V/O relationship was highly significant at all ages ($P<0.0001$, linear regression model), but neither the gradient ($P=0.0961$) nor the intercept ($P=0.649$) changed with age. The volume of intake per proboscis-extension was thus unaffected by the age of the flies.

During the 30 minutes of the combined assay, the flies consumed amounts of blue label that spanned a 30-fold range (equivalent to that found in 5 µg–150 µg food). The food intake of the flies thus varied widely. Despite the variation in the overall amount of feeding, there was no significant variation in the

Table 1. A linear relationship was tested between blue dye accumulations and feeding frequency using ANOVA in linear mixed effects model.

Assay	Fixed effects	P-value	V/O relationship		
			Coefficient	Estimate	Standard error
Dahomey females (NF = 210, NV = 42, 5 trials)	Observation	<0.0001	Intercept	7.91	2.50
			Gradient	42.10	7.99
Dahomey males vs Dahomey females (NF = 200, NV = 40, 4 trials)	Observation	<0.0001	Intercept F	19.53	2.82
	Sex	<0.001	Intercept M	12.83	1.80[b]
	Observation:Sex	not sig.	Gradient	25.81	8.58
Fully fed vs Dietary restriction (NF = 75, NV = 15, 1 trial)[a]	Observation	<0.001	Intercept	15.68	2.40
	Diet	not sig.	Gradient	62.25	16.11
	Observation:Diet	not sig.			
chico heterozygous vs Dahomey control (NF = 90, NV = 18, 3 trials)	Observation	<0.0001	Intercept	4.50	2.96
	Genotype	not sig.	Gradient	55.04	6.02
	Observation:Genotype	not sig.			
takeout^{1} vs Canton-S (NF = 60, NV = 12, 1 trial)[a]	Observation	<0.001	Intercept	1.29	1.25
	Genotype	not sig.	Gradient	62.01	15.78
	Observation:Genotype	not sig.			
ovo^{D} vs $white^{Dahomey}$ (NF = 200, NV = 40, 4 trials)	Observation	<0.0001	Intercept ovo^{D}	56.40	16.48
	Observation:Genotype	<0.001	Intercept w^{Dah}	28.65	8.60[b]
	Genotype	<0.0001	Gradient ovo^{D}	205.52	37.22
			Gradient w^{Dah}	14.46	8.94

The *P* value of the interaction terms is also displayed, which indicated whether the regression coefficients differ between comparative conditions (NF = no. of flies per condition and NV = no. of vials per condition).
[a]These assays were not repeated on different trial dates. The statistical analysis was therefore only on fixed effects, i.e., a regression analysis.
[b]These standard errors are for the differences in the intercepts.
doi:10.1371/journal.pone.0006063.t001

ingestion ratios except in one genotype, ovo^{D1}. The variation in observed food intake is a possible indicator that transferring flies to labelled food may temporarily disturb their feeding behaviour and highlights the importance of measuring feeding during undisturbed conditions if a quantitative measure of normal intake is required. In addition, control-feeding frequency must be measured at the same time as that in the experimental treatments.

Factors that influence feeding during undisturbed conditions

We investigated other variables that could affect food intake during undisturbed conditions. The circadian rhythm is reported to alter feeding in *Drosophila* [27], there may also be an effect from differences in group size, either in a positive (e.g. aggregation behaviour [28]) or negative (e.g. aggressive competition [29]) direction, and finally, dietary composition may also affect feeding.

To test these factors, we performed the undisturbed proboscis-extension assay at 3 different times in the day. Flies are maintained in a 12h: 12h light: dark cycle, and lights-on occurs at 10am and lights-off occurs at 10pm. We performed the proboscis-extension assay in the morning (at lights-on), in the afternoon (4 hours after lights-on), and in the evening (8 hours after lights-on) using 4 different group sizes (1, 2, 5 or 10 flies: **Figure 3a**). Both the time of day and the group size had highly significant effects on the proportion of time spent feeding ($P<0.001$ for both group size and time of day, GLM), while the interaction between these two was not significant ($P = 0.88$). The lowest feeding proportion was observed in the morning for flies housed singly (0.15 of the time spent feeding), and this increased to approximately 0.50 in the afternoon and evening for flies feeding in groups of 5 or more. Both the afternoon and evening feeding proportions were significantly higher than those in the morning ($P<0.0001$ in both cases, GLM). There was no significant difference in feeding proportions between flies during the afternoon and evening ($P = 0.182$, by model simplification). The lowest proportion of feeding was observed for flies housed singly 0.15–0.22 (depending on time of day), and this significantly increased to 0.18–0.31 (depending on time of day) when flies were housed in pairs ($P = 0.009$, GLM). The proportion of flies feeding was found to nearly double when the number of flies was increased to 5 per vial (0.32–0.49, depending on time of day; 2 flies per vial against 5 flies per vial, $P<0.0001$, GLM), and did not increase further when flies were housed at 10 per vial (0.36–0.52, depending on time of day; 5 flies per vial against 10 flies per vial, $P = 0.287$, by model simplification: **Figure 3a**).

Finally, we tested the response of 7-day old female flies to two different yeast-based diets, one made with water-soluble yeast extract (CSYExtract) [15] and the other with lyophilised yeast (SYBrewer's) [7]. The principle difference between these diets is that yeast extract contains only the water-soluble portion of an autolysed yeast culture, whereas the Brewer's yeast product is made of all cell contents and debris after autolysis and pasteurisation. Both of these have previously been used to study the effects of DR [7,15] (**Figure 3b**). The foods 5× CSYExtract

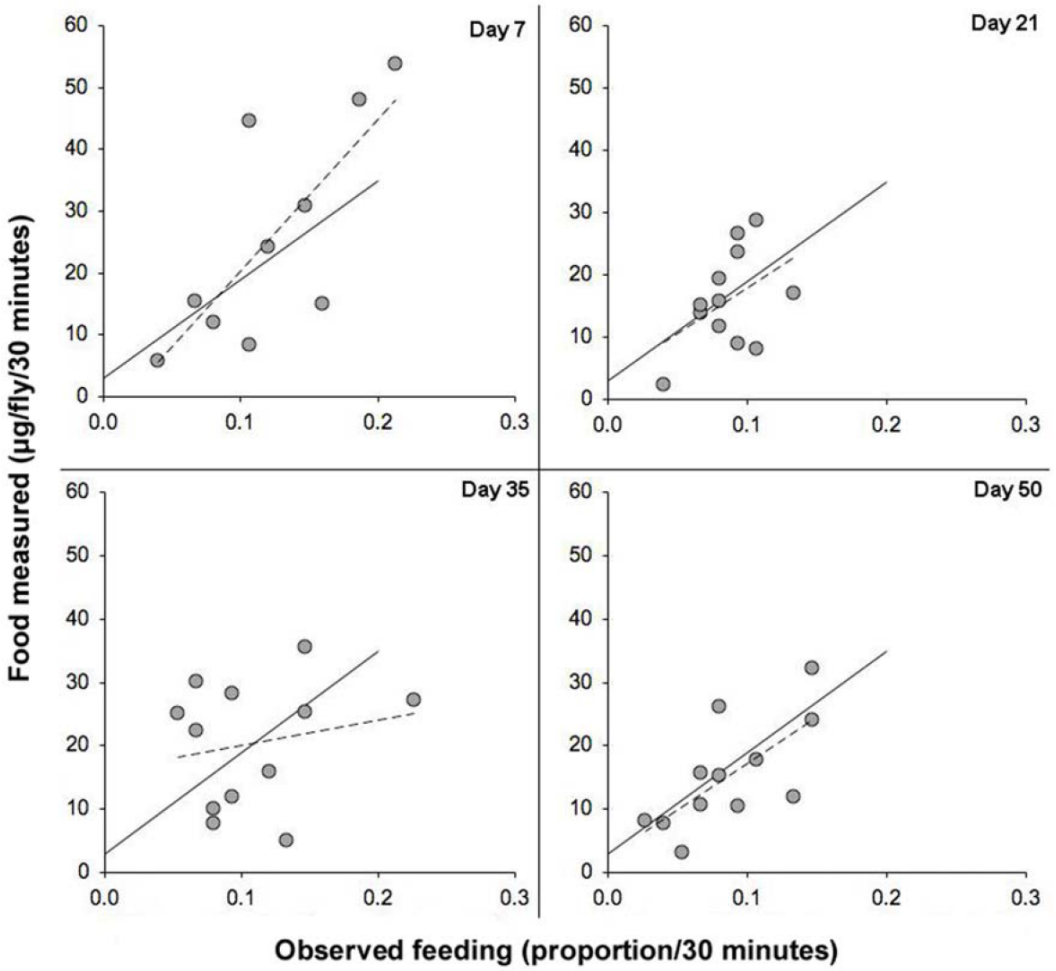

Figure 2. The relationship between blue label uptake and observed feeding events did not change for flies of advancing age. Circles represent measurements of blue label uptake after 30 minutes of feeding and the proportion of feeding events observed during this period. One circle represents one vial containing 5 flies. Experiments were conducted with mated Dahomey females. Assays occurred at 4 different ages: on days 7, 21, 35 and 50 after eclosion. Each assay used 60 flies (12 vials) that were taken from a population that began with 500 individuals. Solid lines represent the significant ($P<0.0001$) V/O relationship with a gradient coefficient of 160.36 (S.E. = 31.39) and intercept of 2.89 (S.E. = 3.45), dashed lines represent the line of best fit for each age class.
doi:10.1371/journal.pone.0006063.g002

and 2× SYBrewer's represent full-fed (FF) conditions, while 1× CSYExtract and 1× SYBrewer's represent DR conditions. The food composition had a significant effect on feeding frequency ($P=0.0126$, GLM). As previously reported, flies exhibited significantly lower feeding frequency when the concentration of yeast extract was increased in the CSYExtract diet (1× CSYExtract against 5× CSYExtract, $P=0.0019$, GLM). In contrast, the feeding frequency of flies was unaffected when altering the yeast concentration of the SYBrewer's diet (1× SYBrewer's against 2× SYBrewer's, $P=0.562$, GLM).

Measuring food intake in lifespan studies

The proboscis-extension method allows repeated feeding assays to be performed with the same cohort of flies, an advantage over methods that sacrifice flies during measurements. As far as we are aware, no publication to date has studied either the feeding frequency of a cohort of flies throughout their lifespan or measured how much food flies consume throughout their lives. This is especially important when monitoring the effects of dietary restriction on lifespan, as the short-term probability of death as revealed by mortality analysis is rapidly affected by changes in nutritional conditions [30]. Thus feeding data from a single time point early in life may not be informative about DR because they do not reflect nutrient intake changes that could occur close to the time of death.

We therefore compared the feeding frequency of once-mated females subjected to DR or control feeding over the course of their lifespan (**Figure 4**). We performed the proboscis-extension assay on cohorts of flies that were kept in a pooled population and assays were performed independently over their lifespan. Feeding declined markedly with the age of the flies, especially during the first 3 weeks of life. The changes in feeding frequency across the lifetime of the flies were significantly different on the two diets (significant interaction between Age and Diet, $P<0.001$, GLM). No overall difference was found in average feeding frequency (0.17 in both cohorts) for the course of the lifespan. However, flies on a

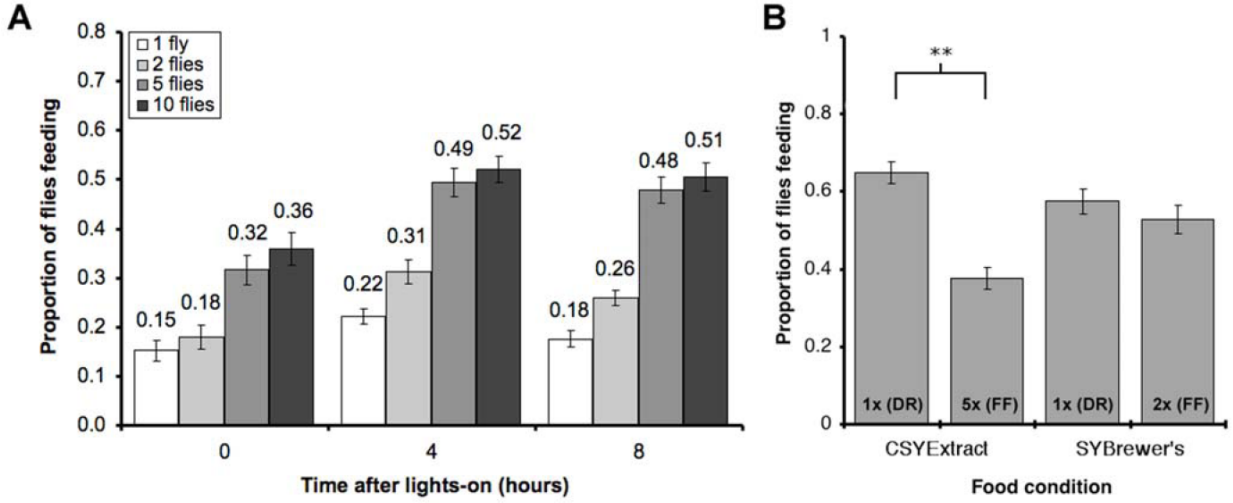

Figure 3. Possible factors that influence feeding frequency. (**a**) The proportion of time spent feeding of 7-day old mated females over a 2-hour period at varying times after lights-on. Females were housed alone, or in groups of 2, 5 or 10 (the number of flies for each condition = 30, with 30 vials for single flies, 15 vials for groups of 2, 6 vials for groups of 5 and 3 vials for groups of 10). We found that increasing the number of flies per vial increased the feeding frequency of each fly, and overall, flies fed more frequently in the afternoon and evening. We calculated the proportion of time spent feeding by summing the scored feeding events divided by the total number of feeding opportunities, which is unaffected by the difference in the number of vials per condition (**b**) The proportion of time spent feeding for flies fed different yeast-based diets. Flies were fed two similar diets containing either a water-soluble yeast extract (CSYExtract) or lyophilised yeast (SYBrewer's) at two different concentrations (DR = Dietary Restriction, FF = Full Fed). While feeding frequency was sensitive to the concentration of yeast extract in the diet, it was unchanged by the concentration of lyophilised yeast (NF = 60 and NV = 12 per condition: ** = $P<0.005$, and error bars = S.E.).
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DR diet fed in a greater proportion of observations than full fed flies early in life, while this reversed later in life when full fed flies fed more than DR flies (between day 31 and day 50), after which the feeding became similar on the two diets. Preliminary studies showed that the feeding frequency of flies on both diets were low at the beginning of the proboscis-extension assay but gradually increased to a steady state over 30 minutes (**Figure 5**).

We also compared the feeding frequency of wild type and long-lived *chico*^{1} heterozygote flies over their lifespans [21]. Reduced *chico*^{1} signalling could lead to a reduction in food intake at some period of life, and therefore increased lifespan through self-imposed DR. Analysis of proboscis-extension over lifetime found that *chico*^{1} heterozygotes fed no more or less than Dahomey at any stage of their lifespan ($P = 0.1639$, GLM). Overall observed feeding proportions also did not differ significantly from wild type controls (*chico*^{1} heterozygotes = 0.259 and Dahomey = 0.283, $P = 0.3193$, GLM: **Figure 6**). As observed before, feeding frequency declined markedly with the age of the flies for both genotypes, and this proved to be significant ($P<0.001$, GLM).

Discussion

In this study, we validated an indirect method of measuring food intake in *Drosophila* (measuring proboscis-extensions) by combining it with a direct method (measuring food intake with a food dye). Despite considerable variation in feeding between replicate groups of flies and between experiments performed on different days, the volume of food ingested per proboscis-extension (ingestion ratio) did not significantly differ between females and males, flies of different ages, flies subjected to DR and flies with mutations in *chico* or *takeout*. Only *ovo*^{D1} females ingested more dye per proboscis-extension.

The observation data revealed that males feed less than females. The higher food intake of female flies is presumably related to their high nutrient-usage in egg-production [31]. The difference in intercept between the two sexes in the combined measurement indicates that amounts of blue dye are always lower in males, although the increase in blue food per proboscis-extension is the same. These lower basal levels of dye may be due to the differences in size (the total volume of the crop and gut), or because the differences in body composition (e.g. fat tissue, vitellogenic material or muscles) may affect the spectrometer reading.

Sterile *ovo*^{D1} females exhibited a greater ingestion ratio than any of the other genotypes tested. This finding is surprising, because egg development is arrested in *ovo*^{D1} flies before the major nutrient investment occurs [32]. If the larger volume of food ingested reflects greater nutrient absorption and utilization in *ovo*^{D1} flies, it could be that they expend more energy through a higher level of activity than fertile flies. This could partially explain why we found a higher ingestion ratio in these mutant flies. *ovo*^{D1} has been reported as feeding less frequently during long-term undisturbed conditions [23], however, our results may not contradict those of Barnes *et al.* (2008), because our data were obtained from the first 30 minutes after transferring to blue-labelled food, and we found no differences with feeding frequency, only with ingestion ratio.

The combined assay is not suitable for long-term, undisturbed feeding experiments because the assay requires that flies are transferred to dyed food, which disturbs fly feeding behaviour. Frequencies of proboscis-extension were observed to be lower than in steady-state conditions, and only reached a constant level by the end of the 30-minute observation period. However, the indirect method alone is accurate for measuring fly feeding during long-term, undisturbed, experimental conditions once assessed by the combined assay for any differences in the ingestion ratio.

Data from the undisturbed steady-state studies suggested that flies exhibit marked diurnal differences in feeding behavior when feeding in groups and earlier in life. The effect of fly group size

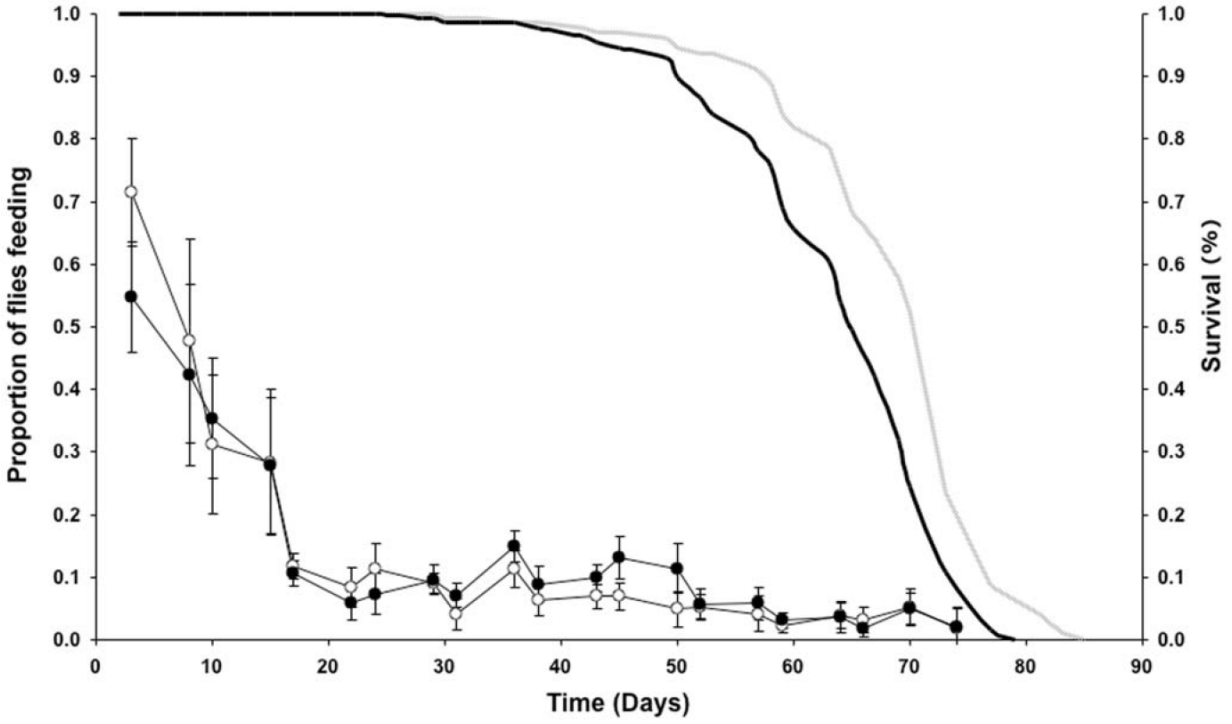

Figure 4. The proportion of time spent feeding for DR (open circles) and full fed (FF) flies (closed circles) on different days of their lifespan. Survivorship curves are indicated with a solid grey line (DR) and a solid black line (FF) flies. Median lifespan: DR = 70 days, FF = 65 days. Proboscis-extension assays used 150 flies (30 vials) per condition. Flies were maintained in populations that began with 1500 individuals per condition (error bars = S.D.).
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may reflect the role of aggregation pheromones, which act as communication signals between flies on breeding substrates, with feeding and oviposition rates increasing with the level of aggregation pheromone [28].

Mutations in the IIS pathway have been shown to extend the healthy lifespan of the nematode worm *Caenorhabditis elegans*, as well as *Drosophila* and the mouse [6,33–35]. Hence, there is intense interest in understanding how the effects of this pathway on healthy

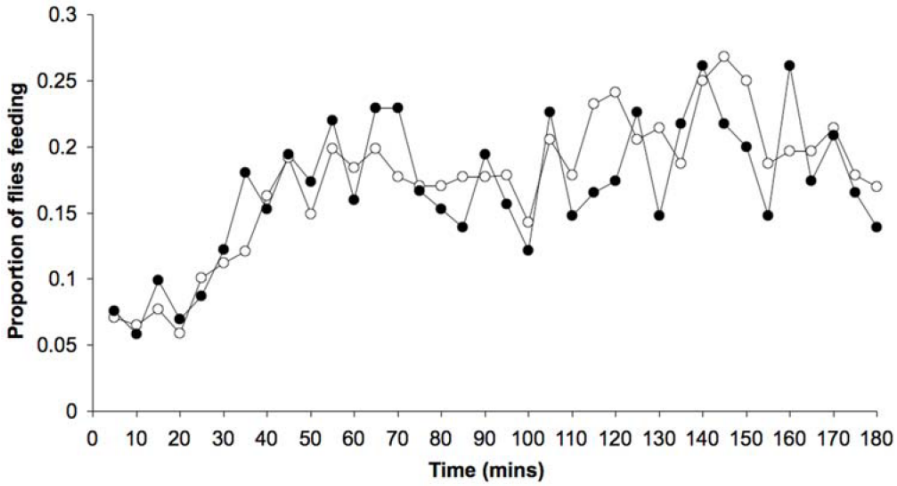

Figure 5. The proportion of time spent feeding during a proboscis-extension assay for DR (open circle) and fully fed (closed circle) once-mated 14-day old females. Flies were maintained on different diets throughout their lifespan. DR females did not differ from fully fed females in feeding frequency. The assay began immediately when the observer arrived. Note the lower proportion of flies feeding during the first 30 minutes of the assay, which may relate to the appearance of the observer in the room (NF = 100; NV = 20).
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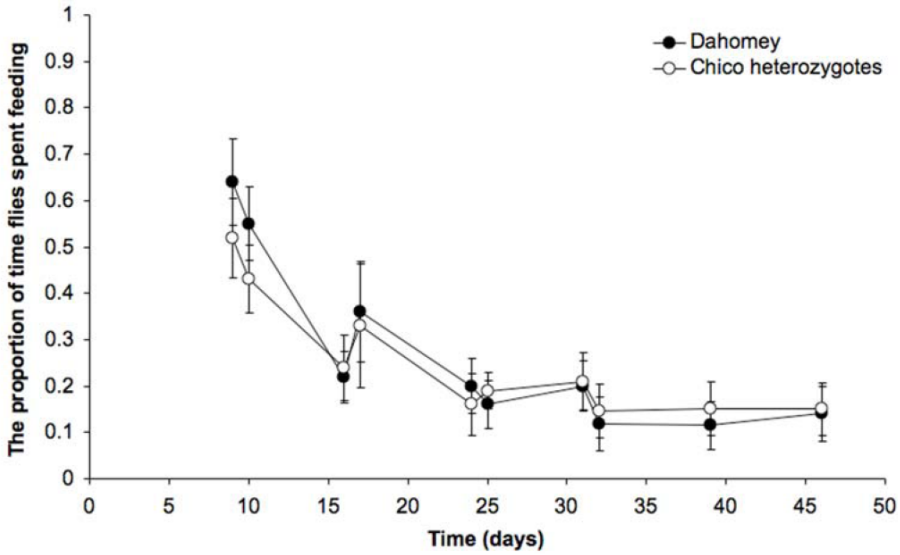

Figure 6. The observed proportion of time spent feeding for Dahomey (control) flies (closed circles) and *chico*^{1} heterozygotes (open circles) on different days of their lifespan, obtained by dividing the number of flies observed feeding by the total number of flies present. Two observers alternately performed assays on the same population of flies. No significant interaction ($P = 0.151$) between the observers' data was found. Assays used 50 flies (10 vials) per condition, flies were maintained in populations that began with 500 individuals per condition; error bars = S.D.
doi:10.1371/journal.pone.0006063.g006

lifespan are mediated. A possible cause for the lifespan-extension effect in flies is that they reduce their food intake, resulting in self-imposed DR. If true, this could also account for the observed overlap between the effects of altered IIS and DR in *Drosophila* [36]. Null mutation of the gene encoding the insulin receptor substrate *chico* in *Drosophila* both extends lifespan [21] and alters the response to DR [37]. We assessed the ingestion ratio and the undisturbed, long term feeding frequency of long-lived *chico*-heterozygotes using the proboscis-extension assay and found total food intake was not reduced in the mutants. The increased survival of *chico*^{1} mutant flies compared to controls can therefore not be explained by a reduction in food intake [21]. Thus the observed extension in lifespan in *chico*^{1} mutants [21] is not simply due to self-imposed DR [30].

DR in flies can be imposed by dilution of their food source, which is available in excess. Flies could therefore adjust their feeding frequency to compensate for the reduction in nutritional value, thus reducing or eliminating the effect of food dilution on nutrient-intake. The literature on this topic is conflicting, with some reports that flies can partially compensate for the food dilution [15], others that they do not [18,19] and others that even report increased food intake with increased nutrition [14]. Although each of these studies examined the effects of DR, none of them employ the same dietary conditions as each other. We therefore tested whether the yeast component of the diet could alter the feeding response to nutrient dilution, by comparing the effects on feeding frequency of DR using SYBrewer's yeast diet with that of a diet used in another published study, CSYExtract [15]. Similar to the data reported by Carvalho *et al.* (2005), we saw feeding frequency decrease as the concentration of CSYExtract in the medium was increased. In contrast, but consistent with previous reports [18,19], flies feeding on the SYBrewer's diet under DR and full fed conditions did not change their feeding frequency. These data demonstrate that different DR recipes can elicit different behavioural responses. This is interesting because it may also mean that different diets affect lifespan-extension in different ways. The flies on SYBrewer's diet fed at the same frequency as flies subjected to DR conditions using CSYExtract, which suggests that flies on the full fed CSYExtract diet decrease their feeding to avoid higher concentrations of food. This is consistent with yeast extract having a toxic effect on flies and shortening lifespan [7].

An important element of studies into ageing is the longitudinal effects of lifespan-altering interventions. Previously, we have reported that flies subjected to DR do not alter their feeding frequency on day 7 of adult life [18]. It is still possible, however, that they do so later in life (day 40 onwards). We therefore conducted a longitudinal study of feeding frequency under DR. Very early in adult life (day 3) DR flies exhibited a higher feeding frequency than those under full fed conditions, but this did not occur over the majority of life and there were even individual instances of higher feeding frequency in full fed flies (later in life) than those subject to DR. This agrees with our previous longitudinal data on feeding frequency under DR [19]. This demonstrates that reduced nutrient intake does indeed correlate with extended lifespan for flies. Our data also show that the level of food consumption in older flies is remarkably lower in comparison to feeding levels in early-life (up to day 14), and more experiments will be required to understand how this lowered nutritional intake may contribute to declining mortality rates observed in late-life [38].

In recent years, various methods have been proposed to measure food intake in *Drosophila* [8,12,15]. However, none of these methods allow *Drosophila* to be measured during conditions that reflect either the experimental set-up they are normally housed in or their feeding frequency in undisturbed conditions. We established that the proboscis-extension method fulfils these criteria.

Methods

Fly stocks and dietary conditions

Wild type Dahomey flies were housed and maintained as described in Bass *et al.* (2007) [7]. The *chico*^{1} allele is maintained as a balanced stock that has been backcrossed to the Dahomey outbred laboratory population as described in Clancy *et al.* (2001) [21]. sn^{w}, ry^{506}, to^{1} (takeout) flies were a gift from Brigitte

Dauwalder. All flies were maintained at 25°C, 65% humidity, on a 12h: 12h light: dark cycle. Unless stated otherwise, all assays used mated females at day 7 after eclosion. Day 7 was chosen because the flies are still young, but several early adult developmental processes have been completed [39]. All flies were reared for assays at a standard density, as for lifespan studies [40], and allowed to mate for 48 h post emergence before being sorted by sex, under light CO_2 anaesthesia, into 30 mL glass vials containing 7 mL food.

The DR food medium contained 100 g autolysed Brewer's yeast powder (MP Biomedicals, Ohio, USA), 50 g sugar, 15 g agar, 30 ml nipagin (100 g/L), and 3 mL propionic acid made up to 1 litre of distilled water. The full fed food contained 200 g autolysed yeast powder, 50 g sugar, 15 g agar, 30 ml nipagin (100 g/L), and 3 ml propionic acid made up to 1 litre of distilled water [7]. In the diet comparison experiment, this medium is labelled SYBrewer's. CSYExtract was made according to [15]. This was made by co-diluting sugar and yeast extract (Bacto Yeast extract, B.D. Diagnostics, Sparks, MD) in a binder of cornmeal (80 g/L), bacto-agar (0.5%) and propionic acid (10 g/L). The 1× concentration contained 10 g/L sucrose and 10 g/L yeast extract.

For DR lifespan experiments, flies were maintained 5 per vial at 25°C, 65% humidity, on a 12h: 12h light: dark cycle. Proboscis-extension assays were performed for 60 minutes at 5-minute intervals, 4 hours after lights-on at 21 separate days across the lifespan experiment.

Proboscis-extension assay during undisturbed conditions

For undisturbed observations of feeding, 7-day-old mated flies of the same sex, were transferred to new food at a density of 5 per vial on the evening before the assay. Flies were maintained in a pooled population, 100 flies per bottle, and a subset was collected and returned before and after the assay. Different measurements on different days were therefore considered to be independent of each other. Vials were coded and placed in a randomised order in rows on viewing racks at 25°C overnight. The assay occurred with minimal noise and physical disturbance to the flies. To avoid recording disturbed fly feeding behaviour, 30 minutes was allowed between the arrival of the observer and commencement of the assay. Observations were performed "blind" the next day for 90 minutes, commencing one hour after lights-on. In turn, each vial was observed for approximately 3 seconds during which the number of flies feeding was noted. A feeding event was scored when a fly had its proboscis extended and touching the food surface while performing a bobbing motion. Once all vials in the experiment had been scored in this way, successive rounds of observations were carried out in the same way for the whole 90 minutes of the assay, which, depending on the size of the experiment meant that each vial was observed once every 2 to 5 minutes. At the end of the assay, the vial labels were decoded and the feeding data expressed as a proportion by experimental group (sum of scored feeding events divided by total number of feeding opportunities, where total number of feeding opportunities = number of flies in vial×number of vials in the group× number of observations). For statistical analyses, comparisons between experimental groups were made on the totals of feeding events by all flies within a vial, to avoid pseudoreplication.

Combined proboscis-extension and blue dye assay

Groups of five 7-day-old mated flies were transferred onto fresh food medium as indicated containing 2.5% (w/v) blue food dye (F D & C Blue Dye no. 1). Vials were scored approximately every 2 minutes for proboscis-extension and after a total of 30 minutes were transferred to eppendorf tubes and snap frozen in liquid nitrogen.

Colour spectrophotometry

Flies were homogenised in 200 µL of distilled water. A further 800 µL of distilled water was added and the suspension passed through a 0.22 µm Millex filter (Millipore Corporation, Bedford) to remove debris and lipids. The absorbance of the liquid sample was then measured at 629 nm [Hitachi U-2001 Spectrophotometer (Lambda Advanced Technology Ltd., UK)]. Age-matched flies exposed to non-dyed food were used as the baseline during spectrophotometry. The amount of labelled food in the fly was calculated from a standard curve made by serial dilution in water of a sample of blue food.

Statistics

Statistical analyses were performed using R, v2.2.1 [41]. To assess the relationship between proboscis-extensions and accumulation of blue dye, a linear mixed effects model was used. This modelled blue dye accumulation as a function of proportion of time observed feeding. Genotype, age and food concentration were specified as fixed effects and trial date as a random effect. To test for non-linearity, a quadratic term of observed feeding events was added to some models. The model fit for the data was reasonably acceptable, judging from residual plots and qq-plots (per trial date). For thoroughness, we re-analysed all models on log-transformed data. Although this further improved the normality of the residuals, the conclusions of the models were qualitatively unaffected.

To compare the effect of time of day, group size and dietary composition on feeding frequency, we used generalised linear models (with binomial error structure and logit link function, the deviances were scaled to correct for over-dispersion, and using *F*-tests for analysing significance). The generalised linear models incorporate information on the sample sizes and use weighted regression analyses. Significance among factor levels (e.g. among the 4 different group sizes) was determined by model simplification, where we evaluated whether combining >1 factor level into a single level led to a significant increase in deviance of the model, using *F*-tests [42]. The same generalised linear models were also used to compare the proportions of time spent feeding in the combined assays.

Author Contributions

Conceived and designed the experiments: RW MDWP LP. Performed the experiments: RW MDWP. Analyzed the data: RW MDWP BW. Contributed reagents/materials/analysis tools: RW MDWP. Wrote the paper: RW.

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